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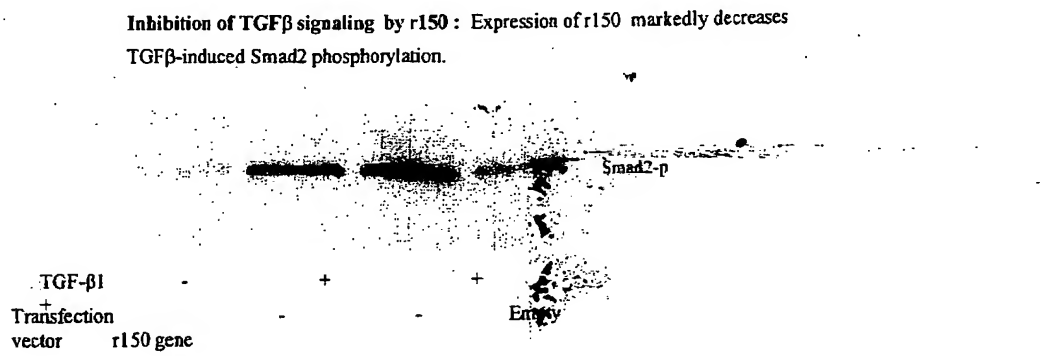
(54) Title: A 150 KDA ACCESSORY RECEPTOR FOR TGF-β.



(57) Abstract: The present invention relates to a TGF-β1 binding protein called r150. This protein has a GPI-anchor contained in r150 itself and not on a tightly associated protein and that it binds TGF-β1 with an affinity comparable to those of the signaling receptors. Furthermore, the released (soluble) form of this protein binds TGF-β1 independent of the types I and II receptors. Also, the soluble form inhibits the binding of TGF-β to its receptor. In addition, evidence that r150 is released from the cell surface by an endogenous phospholipase C is provided. Also, the creation of a mutant human keratinocyte cell line with a defect in GPI synthesis which displays reduced expression of r150 is described. Our results using these mutant keratinocytes suggest that the membrane anchored form of r150 is a negative modulator of TGF-beta responses. These findings, taken together with the observation that r150 forms a heteromeric complex with the signaling receptors, suggest that this accessory receptor in either its membrane anchored or soluble form may antagonize TGF-β responses in human keratinocytes. Experiments with mutants confirmed that TGFβ1 activity can be modulated when the expression of the accessory receptor r150 is silenced. The complete nucleic acid and deduced amino acid sequences are now provided. The r150 cloned nucleic acid was used to study overexpression of r150. When r150 gene is overexpressed, TGFβ responses are increased. r150 and its derivatives or precursors (fragments, variants and nucleic acids encoding the same) will find a broad clinical utility, knowing that TGFβ1 is an important cytokine.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE OF THE INVENTION

A 150 kDa accessory receptor for TGF-β1.

BACKGROUND OF THE INVENTION

Transforming growth factor-β (TGF-β) is a 25 kDa multi functional growth factor which plays a central role in the wound healing process (Roberts and Sporn, 1990; O'Kane and Ferguson, 1997). It is an important regulator of the immune response (Letterio and Roberts, 1998), angiogenesis, reepithelialization (Roberts and Sporn, 1990), extracellular matrix protein synthesis and remodeling (Peltonen et al, 1991; Yamamoto et al, 1994). During wound healing, re-epithelialization initiates the repair process which is characterized by recruitment of epidermal stem cells, keratinocyte proliferation and the formation of an epithelial tongue of migrating keratinocytes at the wound edge (Clark, 1996). TGF-β is chemotactic to keratinocytes and induces the expression of integrins on the migrating epithelium (Hebda, 1988; Zambruno et al, 1995). In spite of its promigratory eff ct on keratinocytes, TGF-β is a potent inhibitor to epithelial cell proliferation *in vitro* (Pietenpol et al, 1990) and *in vivo* (Glick et al, 1993). Targeted deletion of the TGF-β1 gene in keratinocytes causes rapid progression to squamous cell carcinoma (Glick et al, 1994). In addition, the epidermis of transgenic mice expressing a dominant negative TGF-β receptor exhibits a hyperplastic and hyperkeratotic phenotype (Wang et al, 1997). These results support the importance of proper expression of TGF-β and regulation of its function in epidermal development and maintenance of epidermal homeostasis .

TGF-ß is a member of the TGF-ß superfamily which also include activins, inhibins, bone morphogenic proteins, growth differentiation factor 1 (GDF-1) and glial-derived neurotropic growth factor (GDNF) (Kingsley, 1994).

There are three widely distributed TGF-β receptors, type I, type II and type III, all of which have been cloned (Roberts and Sporn, 1990; Massague, 1998). The types I and II receptors are both transmembrane serine/threonine kinases that are essential for TGF-β signal transduction. The type III receptor, also known as

betaglycan, is a high molecular weight proteoglycan that is not required for signaling, but is believ d to play a role in presenting the ligand to the typ II receptor (Lop z-Casillas et al, 1993). Endoglin, is another TGF-β receptor predominantly expressed on endothelial cells (Gougos and Letarte, 1990). According to the present model of TGF-β signal transduction, binding of TGF-β to the type II receptor which is a constitutively active kinase, leads to the recruitment and phosphorylation of the type I receptor (Wrana et al, 1994). The activated type I kinase phosphorylates the central intracellular mediators of TGF-ß signalling known as the Smad proteins (Heldin et al, 1997). Smad proteins can be classified into three groups: the pathway restricted Smads include the Smad2 and Smad3 which are phosphorylated by the type I receptor of TGF-ß or activin, while the Smads 1, 5 and 8 are implicated in BMP signalling. The phosphorylation of the pathway restricted Smads permits their interaction with the common Smad or Smad4 and this heteromeric complex then translocates into the nucleus where it regulates expression of target genes. Finally, there inhibitory Smads which include the Smad 7 and Smad 6 prevent the phosphorlyation of the R-Smads by the type I kinase. (Heldin et al, 1997, Massague, 1998; Wrana and Attisano, 2000)

In blood circulation, TGF-β1 is found bound to the carrier α_2 macroglobulin (α_2M; Webb et al. 1998). α_2M binds many other cytokines and therefore lacks selectivity for TGF- β1. α_2M polymorphism has been associated with Alzheimer's disease, which polymorphism is observed as a deletion in "the bait region" overlapping with TGF- β1 binding domina (Gonias et al. 2000 and Blacker et al 1998).

Although the types I and II receptors are central to TGF-β signaling, it is possible that accessory receptors interacting with the signaling receptors modify TGF-β responses. For example, both endoglin and type III receptor which form heteromeric complexes with the type II receptor have been reported to modulate TGF-β function. When overexpressed in myoblasts, endoglin inhibited while type III receptor enhanced TGF-β responses (Letamendia et al, 1998). In addition, endoglin was shown to antagonize TGF-β mediated growth inhibition of human vascular endothelial cells (Li et al, 2000). Similarly, the newly identified type

I-like receptor BAMBI which associates with TGF-β family receptors can inhibit signaling (Onichtchouk et al, 1999).

There are also a number of molecules that can impact TGF-ß signal transduction by interacting with one or both of the TGF-ß signaling receptors. However, the exact physiological significance of many of these interactions are not clearly defined (for review, Massague, 1998). Three of these interacting proteins: the type II TGF-ß receptor interacting protein (TRIP-1) (Chen et al, 1995), Bα (α subunit of protein phosphatase A) (Griswold-Prenner, 1998), and serine-threonine kinase receptor associated protein (STRAP) (Datta et al, 1998) all contain the highly conserved tryptophan-aspartic acid (WD) repeats. WD domains are important in protein-protein interactions and cellular functions such as cell cycle progression and transmembrane signaling (Neer et al, 1994). TRIP-1 is phosphorylated through its interaction with the type II receptor kinase and exerts an inhibitory effect on TGF-ß induced PAI-1 gene transcription, but has no effect on TGF-ß mediated growth inhibition (Choy and Derynck, 1998). On the other hand, Bα associates with the type I receptor and positively modulates TGF-ß action. Finally, STRAP can interact with both the type I and II receptors and when overexpressed, it exerts an inhibitory effect on TGF-ß mediated transcriptional activation. In addition, STRAP can also interact with the inhibitory Smad7, but not Smad6. STRAP's interaction with Smad7 exerts a stabilizing effect on Smad7's association with the activated type I kinase receptor which prevents Smad2/3's association and subsequent phosphorylation (Datta and Moses, 2000).

The immunophilin, FKBP12, interacts with the TGF-ß type I receptor and acts as a negative modulator of TGF-ß function (Wang et al, 1996). It can interact with unactivated type I receptor and functions to stabilize the quiescent receptor state by protecting phosphorylation sites in the GS domain. Upon ligand stimulation, heteromerization and subsequent phosphorylation of the GS domain by the TGF-ß type II kinase results in the release of FKBP12 (Chen et al, 1997; Huse et al, 1999). In contrast, the TGF-ß type I receptor associated protein-1 (TRAP-1) interacts only with the activated type I receptor kinase (Charng et al, 1998). TRAP-1 is not phosphorylated by the type I kinase and TRAP-1's interaction is reported to

have an inhibitory effect on TGF-ß signaling . However, a recent report describes a different function for TRAP-1 (Wurthner t al, 2001). In this study, TRAP-1 was found to associat with inactive TGF-ß and activin rec ptor complexes and upon ligand stimulation, TRAP-1 is released. The conformationally altered TRAP-1 is then believed to associate and subsequently chaperone Smad4 to the activated Smad2. The α subunit of ras farnesyl protein transferase (FNTA) preferentially interacts with the activated type I receptor and is considered a substrate because it is phosphorylated by the type I kinase and released thereafter (Kawabata et al, 1995). However the functional significance of this phenomenon remains unexplained. The accessory receptors, endoglin and type III receptor which form heteromeric complexes with the type II receptor have also been reported to modulate TGF-ß function. When overexpressed in myoblasts, endoglin inhibited while type III receptor enhanced TGF-ß responses (Letamendia et al, 1998). Glycosylphosphatidyl inositol (GPI)-anchored proteins which lack transmembrane and cytoplasmic domains have also been shown to bind TGF-ß. These proteins have been identified on certain cell lines (Cheifetz and Massague, 1991), but the identity of these GPI-anchored proteins and the role they may play in TGF-ß signaling remain unknown. Recently, the present inventors reported the presence of GPI-anchored TGF-ß binding proteins on early passage human endometrial stromal cells (Dumont et al, 1995), human skin fibroblasts (Tam and Philip, 1998) and keratinocytes (Tam et al, 1998). On human keratinocytes, they identified a 150 kDa GPI-anchored TGF-ß1 binding protein designated as r150 that can form a heteromeric complex with the types I and II TGF-ß receptors (Tam et al, 1998). In addition, they demonstrated that upon hydrolysis fom the cell surface by phosphatidylinositol phospholipase C (PIPLC), the soluble form of r150, retains its ability to bind TGF-ß1 in the absence of the types I and II receptors. In addition, it was demonstrated that the GPI anchor is contained in a protein with a molecular weight of 150 kDa (Tam et al, 2001). This novel GPI-anchored TGF-ß1 binding protein, r150, has the potential to antagonize or potentiate TGF-ß action in keratinocytes. In the absence of the cDNA encoding r150, one way to examine the effect of r150's loss in TGF-ß signaling is to enzymatically release the binding protein by PIPLC treatment prior to testing for alterations in TGF-ß induced responses. However, the efficacy of exogenously added PIPLC is subject to

variability, being affected by pH, temperature, and acylation of GPI-anchored proteins (Shukla, 1982; Chen t al, 1998), thus results obtained may be difficult to interpret. In addition, GPI-anchored proteins that are released may get re-synthesized and re-inserted in the plasma membrane soon after PIPLC hydrolysis. Hence, as an alternative, was have created and isolated a keratinocyte cell line that is mutated in GPI anchor biosynthesis. These cells display a significant loss of r150 from their cell surface, thus allowing a comparative examination of TGF-ß mediated cellular responses in the GPI anchor deficient cell line versus the parental HaCat cells under stable experimental conditions

GPI-anchored proteins lack transmembrane and cytoplasmic domains, and are attached to the cell membrane via a lipid anchor in which the protein is covalently linked to a glycosyl phosphatidylinositol moiety. GPI-anchored proteins have been reported to have roles in intracellular sorting (Rodriguez-Boulan and Powell, 1992), in transmembrane signaling (Brown, 1993) and to associate with cholesterol and glycosphingolipid-rich membrane microdomains (Brown and London, 1998; Hooper, 1999). Also, the GPI anchor enables a protein to be selectively released from the membrane by phospholipases (Metz et al, 1994; Movahedi and Hooper, 1997). r150 was characterized as GPI-anchored, based on its sensitivity to phosphatidylinositol phospholipase C (PIPLC). However, it is important to rule out other possibilities, namely, (i) r150 is not itself GPI-anchored, but is tightly associated with a protein that is GPI-anchored, and therefore is susceptible to release by PIPLC; (ii) r150 is a complex of two lower molecular weight proteins which became inadvertently cross-linked during the affinity labeling procedure.

It is now demonstrated that the GPI-anchor is contained in r150 itself and not on a tightly associated protein and that it binds TGF-β1 with an affinity comparable to those of the signaling receptors. Furthermore, the released (soluble) form of this protein binds TGF-β1 independent of the types I and II receptors. Also, the soluble form inhibits the binding of TGF-β to its receptor. In addition, we provide evidence that r150 is released from the cell surface by an endogenous phospholipase C. Also, a mutant human keratinocyte cell line with a defect in GPI synthesis was created, which display reduc d expression of r150. The results using

these mutant keratinocytes suggest that the membrane anchored form of r150 is a negativ modulator of TGF-beta r spons . These findings, taken together with the observation that r150 forms a heteromeric complex with the signaling receptors, suggest that this accessory receptor in either its membrane anchored or soluble form and its down- or up-regulation may potentiate or antagonize TGF-β responses in human keratinocytes, respectively.

The complete amino acid of a molecule named CD109 was recently disclosed as well as the nucleic acids encoding same (Lin et al. 2002). Sequences comparisons with those of r150 suggest that CD109 is a r150 variant. No definite role has been assigned to CD109 by Lin et al.

SUMMARY OF THE INVENTION

This invention provides a molecule that binds TGF- β1 with a high level of selectivity. This molecule named r150 can be retrieved in a membrane anchored form or as a released free soluble form. Variants and parts of r150 which have the property to bind TGF- β1 are grouped under the name r150-like proteins or peptides. They include those defined in SEQ ID Nos: 2, 4, 8, 10 and 12. Their corresponding coding nucleic acids respectively defined in SEQ ID NOs: 1, 3, 5, 7, 9 and 11.

This invention provides for the use of a protein comprising any one of the following sequences in the making of a medication for inhibiting TGF-β1 activity in a biological tissue SEQ ID Nos: 2, 4, 6, 8, 10 and 12.

Also provided is the use of an antagonist to a protein comprising any one of the following sequences in the making of a medication for increasing TGF-β1 activity in a biological tissue: SEQ ID Nos: 2, 4, 6, 8, 10 and 12.

Also provided is the use of a nucleic acid encoding a protein comprising any one of the following sequences in the making of medication for inhibiting TGF-β1 activity in a biological tissue: SEQ ID Nos: 1, 3, 5, 7, 9 and 11.

Also provided is the use of a molecule which silences the expression of a nucleic

encoding a protein comprising any one of the following sequences in the making of m dication for increasing TGF-β1 activity in a biological tissue: SEQ ID Nos: 1, 3, 5, 7, 9 and 11. Particularly, the sil ncing molecule is an antisense nucleic acid.

The present inventors being the first to elucidate the complete nucleic acid sequence of r150 and of its deduced amino acid sequence, this invention provides an isolated nucleic acid encoding a protein comprising any one of the following sequences : SEQ ID Nos: 2, 4, 6, 8, 10 and 12.

In a specific embodiment, the nucleic acid comprises any one of the following nucleotide sequences: SEQ ID No: 1, 3, 5, 7, 9 and 11.

The nucleic acid is particularly one comprising the nucleotide sequence defined in SEQ ID No: 1.

The above nucleic acids may include crude nucleic acids or recombinant vectors; namely expression vectors capable of governing transcription and translation of the crude nucleic acids inserted downstream to a promotor, are preferred tools for producing r150-like proteins.

Recombinant host cells which comprise the nucleic acids or the recombinant vector are other tools. The vectors are normally selected to comprise sequences compatible with the host's machinery. Intervening sequences located 5' and 3' with regard to the crude nucleic acids are adapted or selected by the skilled artisan desirous to produce a particular type of host cells. The signal peptide may be charged also for another one more appropriate for a given cell type.

There host cells may be domesticated and used in a method of producing a r150-like protein. Such a method comprises the steps of:

- growing a recombinant host cell in a culture medium supporting cell growth and expression of said nucleic acid:
- recovering the protein from the culture medium or from the cell.

The nucleic acids may be antisense nucleic acids. They may be inserted in a recombinant vector, namely an expression vector and recombinant host cells which comprises such antis ns nucleic acids can also be mad .

It is further an object of this invention to provide a TGF-ß1 binding reagent, which comprises a protein comprising any one of the following sequences: SEQ ID Nos 2, 4, 6, 8, 10 and 12.

Compositions of matter which comprise these reagents and a carrier are other objects of this invention.

The carrier may be a pharmaceutical carrier. Otherwise, it may be a solid support to which r150 is bound to immobilize TGF-ß1.

DESCRIPTION OF THE INVENTION

r150 is a TGF-β1 binding molecule. Its complete amino acid sequence as well as the nucleic acid sequence encoding same appear to have been first elucidated by the present inventors. Another group (Lin et al. 2002), using a very different approach (affinity binding to monoclonal antibodies) has found a blood cell surface antigen, which they called CD109. Sequence comparisons show that CD109 (SEQ ID Nos 5 and 6) comprises a 17 amino acid insertion at position 1218-1234 (51 nts). This addition accounts for the difference in amino acids number (1445 for CD109 versus 1428 for r150). Besides that, substitutions of nucleotides are noted: r150 amino acid thr^{1224} is changed for a methionine (CD109 amino acid 1241). CD109 shows polymorphism at residue 703 (Schuh et al. 2002). A tyrosine or a serine represent different alleles of CD109. Such polymorphism would presumably exist for r150. It is possible that CD109 or r150 are responsible for the building of an immune response since allo antibodies are retrieved upon administration of CD109 isoforms. It may therefore be implied that an isoform compatible with the recipient subject's tissue may have to be selected as an administrable r150 active ingredient.

r 150 binds or sequesters TGF-β1, in its membrane anchored form as well as in its free soluble form (SEQ ID Nos: 4 and 8). As a result, TGF- β1

availability is reduced. The effects induced by TGF- β1 are therefore negatively modulated (or inhibited). Such inhibition may be desirable in condition where overproduction of TGF- β1 leads to pathological states (cancer is a specific xample of such pathology). On the contrary, in other occasions, increasing TGF- β1 activity may be sought. For example, TGF- β1 encourages tissue or organ graft success. Therefore silencing r150 would have for effect to increase TGF- β1 availability and increase graft success.

r150 further appears to be related to α_2 macroglobulin (α_2M) and since the TGF- β binding domain has been determined for α_2M by Webb et al. (1998), the corresponding domain in r150 is presumed to be located a region corresponding to α_2 macroglobulin amino acids 666-706. These corresponds to r150 amino acids 651-683 (SEQ ID No: 10). Therefore, the r150 peptide having the sequence defined in SEQ ID NO: 10 is also contemplated as a TGF-β1 binding peptide within the scope of the invention; the nucleic acid encoding this peptide is as well.

Webb et al. (2000) even found the minimal α_2M TGF-β1 binding sequence which appears to be delineated by amino acid 717 and 733. The corresponding strectch in r150 is found between amino acid residues 694 and 712 (SEQ ID No.12).

Gomas et al. (2000) reported that α_2M gene polymorphism has been associated with Alzheimer's disease, which polymorphism is observed as a deletion in "the bait region" overlapping with TGF-β1 binding domain. It is envisageable that r150 could be useful to sequester and neutralize TGF- β1 especially in diseases wherein α_2M is deficient. Any portion of r150 or variants thereof that is capable of binding TGF- β1 activity is intended to be used in the making of a medication or a method or composition for inhibiting TGF-β1 activity. This includes proteins or peptides comprising sequences defined in SEQ ID NOs. 2, 4, 6, 8, 10 and 12. These r150-like proteins or peptides would include any molecule having at least 50% homology with the above sequences. On the opposite, any molecule having an antagonistic activity to the above r150-like proteins or peptides would find a use in

th making of a medication or a method or a composition for increasing TGF-β1 activity.

Nucleic acids encoding the above r150-like proteins or peptides represent an alternative to the direct administration of proteins or peptides. Antisense nucleics would on the opposite silence the expression of r150-like proteins or peptides. All these nucleic acids include recombinants vectors, namely expression vectors, which are available and well known to the skilled artisan.

A very large body of literature describes diseases or disease models involving up and down regulation of TGF-β1 activity. Nowadays, TGF-β1 binding proteins decorin and an anti-TGF antibody are currently under clinical trials. The present r150-like proteins or peptides could represent a valuable and advantageous alternative to these molecules, because of their selectivity for TGF-β1 isoform, combined to their hydrosolubility.

Here is a non-exhaustive list of disease models where alteration of TGF-β action has been shown to be of therapeutic benefit:

Cancer progression:

Note: TGF-β has biphasic effects during tumorigenesis, acting early as a tumor suppressor, but later stimulating cancer progression.

(i) Suppression of tumor progression by TGF- β

Akhurst R.J. and Derynck R. (2001). TGF-β signaling in cancer a double-edged sward. TRENDS in Cell Biology 11: S44 – S51.

Welch, Dr. et al (1990). Transforming growth factor- β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc. Natl. Acd. Sci. USA 87: 7678-7682

Markowitz, S. et al. (1995) Inactivation of the type II TGF- β receptor in colon cancer cells with microsatellite instability. Science 268,1336–1338.

Massagué, J. et al. (2000) TGF- β signaling in growth control, cancer, and heritable disorders. Cell 103, 295–309.

(ii) Stimulation of tumor progression by TGF- β

Hojo, M. et al. (1999) Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 397, 530–534.

Yin, J.J. et al. (1999) TGF- β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J. Clin. Invest. 103, 197–206.

Exogenous TGF- β1 promotes wound healing where as inhibiting TGF- β1 activity or enhancing TGF- β3 activity reduces scarring in animal models

Roberts, A.B., and Sporn, M.B. (1996). Transforming growth factor beta. In: The molecular and cellular biology of wound repair (second edition), Clark, R.A.F. (ed), Plenum Press, New York, p275-308.

Mustoe, T.A., Pierce, G.F., Thomason, A., Gramates, P., Sporn, M.B., and Deuel, T.F. (1987). Accelerated healing of incisional wounds in rats induced by transforming growth factor- β. Science 237: 1333-1336.

Quaglino, D., Nann y, L.B., Ditesheim, J.A., and Davidson, J.M. (1991). Transforming growth factor-β stimulates wound h aling and modulates extracellular matrix gene expression in pig skin: incisional wound model. J. Invest. Dermatol. 97: 34-42.

O'Kane, S., and Ferguson, M.W.J. (1997). Transforming growth factor- βs and wound healing. Int. J. Biochem. Cell. Biol. 29:63-78.

Shah, M., Foreman, D.M., and Ferguson, M.W.J. (1995). Neutralization of TGF- β1 and TGF- β2 or exogenous addition of TGF- β3 to cutaneous rat wounds reduces scarring. J. Cell Science 108: 985-1002.

Choi, B-M., Kwak, H-J., Jun, C-D., Park, S-D., Kim, K-Y., Kim, H-R., and Chung, H-T. (1996). Control of scarring in adult wounds using antisense transforming growth factor- β1 oligodeoxynucleotides. Immunol. Cell Biol. 74:144-150.

Blocking TGF- β1 overproduction reduce tissue fibrosis (pulmonary fibrosis, liver cirhosis, glomerulonephritis, scleroderma and atherosclerosis).

Border et al, (1990). Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. Nature 346 (6282): 371-374

Isaka Y., Brees D.K., Ikegaya K., Kaneda Y., Imai E., Noble N.A., Border W.A. (1996). Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat. Med. 2 : 418–423.

Isaka Y., Akagi Y., Ando Y., Tsujie M., Sudo T., Ohno N., Border W.A., Noble N.A., Kaneda Y., Hori M., and Imai E. (1999). Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular

matrix accumulation in experimental glomerulonephritis [s e comm nts]. Kidney Int. 55 :465–475.

Khalil, N. and Greenberg AH (1991). The role of TGF- β in pulmonary fibrosis. Ciba Found Symp. 157: 194-207.

Yamamoto, T., Takagawa S., Katayama I., and Nishioka K. (1999). Anti-Sclerotic effect of transforming growth factor –beta antibody in a mouse model of bleomycin-induced scleroderma. Clin Immunol, 92(1):6-13.

Gressner, A.M., Weiskirchen, R., Breitkopf K., and Dooley S. (2002). Roles of TGF-beta in hepatic fibrosis. Front Biosci (7): d793-807.

Sheppard D. (2001). Integrin-mediated activation of transforming growth factor-beta(1) in pulmonary fibrosis. Chest 120(1 Suppl):49S-53S.

McCaffrey, T.A. (2000). TGF- βs and TGF-β receptors in atherosclerosis. Cytokine. Growth Factor Rev. 11: 103-114.

TGF-beta has tissue protective effects (against ischemia reperfusion injury) in the heart, brain and kidney.

Lefer AM., Ma X-L, Weyrich AS, Scalia R. (1993). Mechanism of the cardioprotective effect of TGF- β1 in feline myocardial ischemia and reperfusion. Proc. Natl. Acad. Sci. USA 90: 1018-1022.

Lefer AM, Tsao P, Aoki N, Palladino MA. (1990). Mediation of cardioprotection by transforming growth factor-β. Science 249: 61-64.

McNeill H, Williams C, Guan J, Dragunow M, Lawlor P, Sirimann E, Nikolics K,

Gluckman P. (1994). Neuronal rescue with transforming growth factor-beta 1 after hypoxic-ischaemic brain injury. Neuroreport 5: 901-904.

Mehta JL, Yang BC, Strates BS, Mehta P. (1999). Role of TGF-beta1 in platelet-mediated cardioprotection during ischemia-reperfusion in isolated rat hearts. Growth Factors 16: 179-190.

Recombinant hosts comprising the above nucleic acids or recombinant expression vectors can be used as a biological machinery in the production of the r150-like proteins or peptides. The elucidation of the nucleic acid sequence of r150 therefore leads to a method of producing these proteins or peptides by recombinant technology.

A variety of TGF-β1 binding reagents and compositions may be derived from the present invention.

First, peptides such as those defined in SEQ ID. Nos: 10 and 12 may be used as such as a TGF-β1 binding reagent. Larger molecules like those defined in SEQ ID Nos 2, 4, 6 and 8 could be conjugated (through their anchoring region) to a carrier. The carrier may take the form, for example, of a solid or semi-solid medium (beads, chromatography columns, plates, etc.), to immobilize TGF-β1. Pharmaceutical compositions would take any suitable form, depending on th selected route of administration. A r150-like protein or peptide (SEQ ID Nos: 2, 4, 6, 8, 10 and 12) would be formulated with a pharmaceutically acceptable carrier. Doses equivalents those used by intravenous route for decorin and/or the TGF-antibody can be produced.

Objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Affinity cross-link labeling of human neonatal keratinocytes with ^{125}I-TGF-β1. Confluent monolayers were affinity labeled with 100 pM ^{125}I-TGF-β1 in the absence or presence of unlabeled TGF-β1, -β2, or -β3. Solubilized cell extracts were analyzed by SDS-PAGE under non reducing conditions and autoradiography **(a)**. Competition curves for r150 and the types I and II TGF-β receptors were derived by densitometric analysis of a typical autoradiogram. The data for each binding complex are expressed as a percent of the value in control wells incubated with ^{125}I-TGF-β1 alone and are plotted against the concentration of unlabeled TGF-β1 **(b)**, -β2 **(c)**, or -β3 **(d)**. The autoradiogram and competition curves are representative of three different experiments.

Figure 2: Temperature induced phase separation in Triton X-114 of TGF-β binding proteins on human neonatal keratinocytes. Keratinocytes affinity labeled with ^{125}I-TGF-β1 were lysed in 1% Triton X-114. The Triton X-114 soluble material was incubated at 30°C for 10 minutes followed by centrifugation at room temperature to induce phase separation of the detergent rich phase and the aqueous detergent poor phase. **A**: An aliquot (20%) from each phase was precipitated with ethanol/acetone, and analyzed by SDS-PAGE under reducing conditions. ^{125}I-TGF-β1 labeled proteins in the aqueous **(Aq)** and the detergent rich **(Det)** phases, representative of three different experiments, are shown. **B:** The remaining 80% of the detergent phase was utilized to determine the effect of PIPLC treatment on the partitioning of r150 in Triton X-114. The detergent phase was incubated in the absence **(-)** or presence **(+)** of 0.6 U/ml of PIPLC followed by temperature induced phase separation and ethanol/acetone precipitation as above, to distinguish between the hydrophilic and amphipathic forms of the proteins. Analysis of the ^{125}I-TGF-β1 labeled proteins in the aqueous phases of PIPLC treated (+) and untreated (-) samples, by SDS-PAGE under reducing conditions are shown. The results shown are representative of two different experiments.

Figure 3: (a) Affinity labeling of soluble r150 with ^{125}I-TGF-β1. To verify that the soluble form of the r150 can bind to TGF-β1, human keratinocytes (HaCaT) were left untreated (-) or treated with 0.6 U/ml of PIPLC (+). The GPI-anchored proteins released into the supernatant were concentrated and an aliquot

was affinity cross-link labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β1 and subjected to SDS-PAGE under reducing conditions. The result shown is representative of four different experiments. **(b)** Inhibition of ^{125}I-TGF-β1 binding to TGFβ receptors by soluble r150. Confluent monolayers of HaCaT cells grown in T-25cm^2 culture flasks were left untreated or treated with 0.6 U/ml of PIPLC for 60 minutes at 37°C. The supernatants were collected and concentrated by Centricon. MvLu1 cells were affinity labeled with 50 pM ^{125}I-TGF-β1 in the absence or presence of indicated doses of supernatants and ^{125}I-TGF-β1 specifically bound was plotted as a function of the amount of supernatant used. The arbitrary unit of "1" is equivalent to a dose of supernatant from a T-25cm^2 flask (approximately 1 x 10^6 cells). **(c)** Effect of anti-TGF-β1 on the inhibition of TGF-β1 binding to TGF-β receptors. Confluent monolayers of MvLu1 cells were affinity labeled with 50 pM ^{125}I-TGF-β1 in the absence (C), or presence of PIPLC treated supernatant (+PIPLC - S), or PIPLC-S pretreated with non-immune rabbit IgG (15µg/ml), or PIPLC-S pretreated with anti-TGF-β1 antibody (15µg/ml). To demonstrate that the anti-TGF-β1 antibody effectively neutralizes TGF-β, experiments were also performed with 100 pM of TGF-β1 (+β1), β1 pretreated with non-immune rabbit IgG (15µg/ml), or β1 pretreated with anti-TGF-β1 antibody (15µg/ml). The values shown in (b) and (c) are the mean (+/- S.D.) of at least three to five different experiments.

Figure 4: Identification of a GPI-anchor in r150. Human neonatal keratinocytes were harvested and treated with PIPLC (as described in Materials and Methods). The supernatant containing the GPI-anchored proteins were purified using a TGF-β1 affinity column (see Materials and Methods for details). After the addition of the sample to the column, 0.5 ml fractions were collected during washing and elution. **(a)** An aliquot from each fraction was affinity labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β1 and samples were analyzed by SDS-PAGE under reducing conditions. Only fraction 21 demonstrated an affinity labeled protein at 150 kDa while in adjacent fractions no 150 kDa band was detectable. Affinity labeling pattern obtained for fraction 21 and fraction 18 are shown. **(b)** Selected fractions were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and the samples were immunoblotted with an anti-CRD antibody (Oxford GlycoSystems). A 150 kDa protein was detected in

fraction 21 but not in adjacent fractions (fraction 18). Immunoblotting with the anti-CRD antib dy was performed twice and the affinity cross-link labeling experiments of solubl r150 was done at least three times.

Figure 5: Immunoprecipitation of affinity labeled TGF-β binding complexes on human neonatal keratinocytes with the anti-CRD antibodies. Keratinocytes not treated with PIPLC were affinity labeled with 100 pM ^{125}I-TGF-β1 **(a & b)** or ^{126}I-TGF-β2 **(c)** and were not immunoprecipitated (nip) or subjected to immunoprecipitation with an anti-CRD antibody against trypanosomal sVSG (Oxford GlycoSystems) **(a & c)**, or with an anti-CRD antibody against porcine membrane dipeptidase (Broomfield and Hooper, 1993) **(c)**. In the lane marked anti-CRD+peptide the immunoprecipitation was carried out using the anti-CRD antibody which was preincubated with PIPLC treated membrane dipeptidase. Immune complexes were subjected to SDS-PAGE under reducing conditions and analyzed by autoradiography. The results shown are representative of at least four to five experiments.

Figure 6 A: Expression of CD59 is decreased in keratinocytes mutated in GPI anchor biosynthesis. A representative histogram of the expression of CD59 in a GPI anchor deficient clone, GPI M (white columns) and parental HaCat cells (black columns) as assessed by flow cytometry using an anti-CD59-FITC labeled antibody. The immunoflourescence intensity of CD59 in the GPI anchor deficient cells was approximately 50% to that of the HaCat cells. The control performed in the absence of antibody is also included (doted peak). Flow cytometry was performed at least three times.

Figure 6B: Cell surface expression of r150 is markedly decreased in GPI anchor mutated cells. Confluent monolayers of HaCat and GPI M cells were affinity labeled with 100 pM ^{126}I-TGF-ß1. Solubilized cell extracts were analyzed on SDS-PAGE under reducing conditions.

Figure 7 A: Doubling time curves of HaCat and GPI anchor mutated cells. HaCat and GPI M cells were seeded at 8.0 x 10^5 cells in 60 mm dishes

in duplicat and the cell number for each was determined at 14, 24 28, 32, 36, 48, 52 and 72 hours using a heamocytometer.

Figures 7 B and C: Th cellular morphology of GPI anchor mutated cells is identical to that of HaCat cells. Microscopic representation (10x magnification) of HaCat (Fig. 7B) or GPI M cells (Fig. 7C) under normal culture conditions.

Figures 8 A and B: Enhanced TGF-ß stimulated transcriptional response in GPI anchor mutated cells. HaCat and GPI M cells were transiently transfected with 1 µg of p3TP-Lux reporter gene construct and were left untreated or treated under serum free conditions with the indicated concentrations of TGF-ß1 for 4hrs (Fig. 8A) or 16 hrs (Fig. 8 B). The luciferase activity was normalized to ß-galactosidase activity expressed from a co-transfected CMVßgal plasmid . The data is representative of at least three independent experiments. Error bars represent standard deviation.

Figures 9 A and B: GPI anchor mutated cells display enhanced responses at low doses of TGF-ß1. HaCat and GPI M cells were left untreated or treated with the indicated doses of TGF-ß1 (Fig. 9 A) and TGF-ß2 (Fig. 9 B) und r serum free conditions for 20 minutes. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated form of Smad2 . Immunoblotting was repeated with an anti-Smad2 antibody that recognizes total Smad2 (unphosphorylated and phosphorylated forms) or an anti-STAT3 antibody to demonstrate equal protein loading. This data is representative of three different experiments.

Figures 10 A, B and C: Elevated Smad2 phosphorylation is sustained in GPI anchor mutated cells. HaCat and two GPI anchor deficient clones GPI M (Fig. 10 A) and GPI M1 (Fig. 10 B) or a keratinocyte clone that was not GPI anchor mutated, GPI NM (Fig. 10 C) were treated with 100 pM TGF-ß1 for the indicated times. The control lane (-) received no TGF-ß1 treatment. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated form of Smad2. The same nitrocellulose membrane was reblotted and with an anti-STAT3

antibody to demonstrate equal protein loading. Thi data is repesentative of at least three different xp riments.

Figure 11: Autophosphorylation of type II kinase in HaCat and GPI anchor mutated cells. HaCat or GPI M cells were left untreated (-) or treated (+) with 100 pM of TGF-ß1 for 20 minutes. Precleared lysates were immunoprecipitated with 3 µg/ml of anti-type II receptor antibody overnight. Following adsorption to protein A sepharose beads, 10 µCi of gamma ^{32}P was added to the immunocomplexes and incubated for 30 minutes at 30°C to allow phosphorylation to occur. The reaction was halted by the addition of sample buffer and immunocomplexes were then subjected to SDS-PAGE under reducing conditions. This data is representative of two different experiments.

Figure 12: Schematic diagram representing the cloned sequence of the r150 protein.

Figure 13: HaCaT or 293 cells were transfected with r150 gene or the empty vector (pCMV sport 6) and cell lysates were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane and immunoblotted with anti-CRD antibody. The Western blot shown is representative of four experiments (two each with HaCaT and 293 cells).

Figure 14: HaCaT or 293 cells were transfected with r150 gene or the empty vector (pCMV sport 6) or were left untransfected. Cells were allowed to recover for 24 hrs and were treated with 100 pM TGF- 1 for 30 minutes. Cell lysates were then Western blotted with an anti-phosphoSmad2 antibody.

Figure 15: HaCaT or 293 cells were transfected with r150 gene or the empty vector (pCMV sport 6) or were left untransfected. Cells were allowed to recover for 24 hrs and were treated with 100 pM TGF- 1 for 24 hours, or were left untreated. The luciferase activity was normalized to glactosidase activity obtained from a cotransfected CMV gal palsmid.

Figure 16: Schematic model of the mechanism by which r150 inhibits TGF- r sponses.

Figure 17: Sequence of CD109, published by another, representant r150 putative variants. (Genbak accession number AF410459 and AAL84159.1.)

Figure 18: Alignment of α_2-macroglobulin and r150 partial sequences.

EXAMPLE 1:

CHARACTERIZATION OF TGF-β1 BINDING PROTIEN DIFFERENT FROM OTHER TGF-β RECEPTORS

METHODS

Cell Culture:

Neonatal keratinocytes were prepared from foreskin tissue obtained at newborn male circumcision as described by Germain et al (1993). Th keratinocytes were cultured in keratinocyte serum free medium (Gibco, Burlington, Ontario) and cells of third to fifth passage were used for experiments. The immortalized keratinocyte cell line, HaCaT, was obtained from Dr. Boukamp (Heidelberg, Germany), and the mink lung epithelial cells (Mv1Lu) were from ATCC. Both cell types were maintained in Dulbecco's Minimal Essential Medium (D-MEM) supplemented with 5% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (Gibco, Burlington, Ontario). All cells were maintained at 37°C in an atmosphere of 5% CO_2/air.

Affinity labeling of cells:

Iodination of TGF-β1 (Collaborative Biomedical) was done as described (Philip and O'Connor-McCourt, 1991). Affinity labeling technique was performed as detailed previously (Dumont et al, 1995). Briefly, cell monolayers were washed with ice-cold binding buffer (D-PBS or Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+}, pH 7.4) containing 0.1% bovine serum albumin (BSA). Cells were incubated with 100- 200pM of ^{125}I-TGF-β1 for three hours at 4°C. In some experiments, incubations were done in the absence or presence of increasing

concentrations of unlabeled TGF-β isoforms to determine the competition profiles of the receptors. Th receptor-ligand complexes were cross-linked with 1mM Bis-(Sulfosuccinimidyl) suberate (BS^3, Pierce). The reaction was stopped by the addition of glycine and the cells were solubilized, and separated on 3-11% polyacrylamid SDS gel. The results were analyzed by using autoradiography followed by quantitative densitometry (Gel-Cypher, Lightools Inc, Encinitas,CA or ImageQuant, Molecular Dynamics, Sunnyvale, CA)

Temperature induced phase separation in Triton X-114 of r150 and hydrolysis by PIPLC:

Temperature induced phase separation in Triton X-114 and PIPLC treatment was performed as described previously with modifications (Bordier, 1981). Keratinocytes were affinity labeled with 150 pM of ^{125}I-TGF-β1 and lysed in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% Triton X-114, 1 mM phenylmethylsufonyl fluoride and protease inhibitor cocktail (200 µg/ml BSA, 1 µg/ml leupeptin, 10 µg/ml benzamide, 10 µg/ml soyabean trypsin inhibitor, and 2 µg/ml pepstatin) for 60 minutes at 4°C. The cell lysates were centrifuged at 13 000 x g for 15 minutes at 4°C. The Triton X-114 soluble material was incubated at 30°C for 10 minutes followed by a 10 minute centrifugation at 13 000 x g at room temperature to separate the detergent rich phase from the aqueous detergent poor phase. An aliquot (20%) from each phase was precipitated with ethanol/acetone, and analyzed by SDS-PAGE and autoradiography. The remaining 80% of the detergent phase was utilized to determine the effect of PIPLC on the detergent solubility of r150 using the method of Lisanti et al (1988). Briefly, the GPI-anchored protein enriched detergent phase was incubated with or without 0.6 U/ml of PIPLC (Roche Diagnostics) for one hour at 37°C with mild agitation. Temperature induced phase separation was then repeated. Both the aqueous and detergent phases were precipitated by adding ethanol/acetone, and subjected to SDS-PAGE and autoradiography.

Affinity labeling of soluble r150:

Neonatal keratinocytes were harvested by treating confluent monolay rs with Hanks' balanced salt solution containing 5 mM EDTA (pH 7.5). The

cell pellet was washed with D-PBS and treated with 0.6 U/ml of PIPLC or left untreated, for one hour at 37°C with mild agitation.

The supernatant containing the released GPI-anchored proteins was collected and concentrated by Centricon 30 (Amicon). Aliquots of the concentrated supernatant were affinity labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β (7.5 nM) and analyzed by SDS-PAGE as described above except that the solubilization step was omitted.

^{125}I-TGF-β1 binding to Mv1Lu cells

To test whether soluble r150 regulates the binding of TGF-β to its receptors, the supernatant obtained from PIPLC treated HaCaT cells were used in a ^{126}I-TGF-β1 Mv1Lu binding assay. The HaCaT cells, which display the r150 with identical properties as the neonatal keratinocytes (Tam et al, 1998), were grown in T-25cm^2 tissue culture flasks (Falcon) were left untreated or treated with PIPLC, and the resulting supernatants were concentrated by Centricon 30 (Amicon). Mv1Lu cells were incubated with 50 pM of ^{125}I-TGF-β1 in the absence or presence of increasing doses of the concentrated supernatant for three hours at 4°C. The cells were washed, solubilized and the bound radioactivity was determined by a gamma counter.

To rule out the possibility that any alteration in ^{125}I-TGF-β1 binding caused by the supernatants was not due to the presence of TGF-β1, the above binding assay was also done in the presence of the supernatant treated with an anti-TGF-β1 antibody. The supernatant was incubated with the antibody (15 µg/ml) overnight at 4°C. It was then precleared of immune complexes and excess antibody that may interfere with the assay, by incubating with a protein A Sepharose slurry (PharmaciaBiotech) for two hours before addition to the assay. The TGF-β1 antiserum (obtained from Dr.M. O'Connor-McCourt ; Moulin et al, 1997) was purified on a HiTrap Protein G column (PharmaciaBiotech) following standard procedures. The specificity of the antibody was verified by Western Blot analysis

Determination of TGF-β concentration in HaCaT supernatant:

The concentration of TGF-β1 was quantitated in the supernatants from HaCaT cells left untreated or treated with PIPLC using a mink lung pithelial cells-luciferase assay as described by Nunes et al (1996). This quantitative bioassay for TGF-β is based on the ability of TGF-β to induce the expression of plasminogen activator inhibitor type 1 (PAI-1) gene. The mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the luciferase reporter gene were provided by Dr. D.B. Rifkin (New York University Medical Center). Briefly, these cells were incubated with varying doses of HaCaT supernatant, and the luciferase activity (expressed as relative light units) was quantitated by a Berthold Luminometer. Recombinant TGF-β1 (Austral Biochemicals; 0.5 pM-50 pM) was used to create a standard curve.

Immunoaffinity Chromatography and Immunoblotting of r150:

Neonatal keratinocytes were harvested by treating confluent monolayers with Hanks' balanced salt solution containing 5 mM EDTA (pH 7.5). The cell pellet was washed with D-PBS and treated with 0.6 U/ml of PIPLC for one hour at 37°C with mild agitation. The supernatant containing the released GPI-anchored proteins was purified through a TGF-β1 affinity column (made available by Dr. M. O'Connor-McCourt, Montreal, Quebec). The column was prepared by incubating one milligram of TGF-β1 in a 200 mM HCO_3 (pH 8.3) buffer containing 30% (v/v) of n-propanol with five milligrams of Reacti-gel (Pierce) for 72 hours at 4°C. The reaction was stopped by the addition of 200 µl of 2 M TBS (pH 7.4), and the gel was washed to remove any unbound TGF-β1. The supernatant containing the GPI-anchored medium was loaded on the column equilibrated with 50 mM Tris (pH 7.4) and eluted with a 10 mM citrate/300 mM NaCl buffer (pH 2.5). Fractions of 0.5 ml were collected, and each fraction was analyzed for binding to TGF-β1, and immunoblotted for the presence of GPI anchor.

To verify binding to TGF-β1, an aliquot from each fraction was affinity labeled with 150 pM of ^{125}I-TGF-β1 and analyzed by SDS-PAGE and autoradiography as described above, except that the solubilization step was omitted.

The fraction containing r150 and adjacent fractions were immunoblotted with the anti-CRD antibody to detect the GPI anchor. The anti-CRD antibody is specific to the inositol 1,2 cyclic monophosphate moiety, known as the "cross-reacting determinant" (CRD) which is exposed in GPI-anchored proteins that have been hydrolyzed by PIPLC. The antibody obtained from Oxford GlycoSystems (Wakefield, MA), was raised against the inositol 1,2 cyclic monophosphate moiety of the trypanosome variant surface glycoprotein (VSG). Samples were analyzed on 3-11% polyacrylamide gradient SDS gels and transferred to nitrocellulose membrane. The membrane was blocked in TBS-T (30 mM Tris, 150 mM NaCl, pH 7.5, 0.5% Tween 20) containing 5% non-fat dry milk and was incubated overnight with 4 µg/ml of the anti-CRD antibody at 4°C. The blots were washed in TBS-T and incubated for three hours at room temperature with the alkaline phosphatase conjugated secondary antibody (1:1500) (Roche Diagnostics). The membrane was then subjected to chemiluminescence analysis (CDP-Star) as detailed by the manufacturers (Roche Diagnostics).

Immunoprecipitation of r150:

Two different anti-CRD antibodies (i) Oxford GlycoSystems antibody and (ii) an antibody raised against a mammalian GPI-anchored pig membrane dipeptidase (MDP) were used for immunoprecipitation studies. The latter antibody was isolated from the bulk of the anti-MDP antiserum by fractionation on a column of the immobilized form of the trypanasome variant surface glycoprotein. Both anti-CRD antibodies are specific to the inositol 1,2-cyclic monophosphate and have been well characterized (Zamze et al, 1988; Broomfield and Hooper, 1993). Cells were affinity labeled with 200 pM ^{125}I-TGF-β1 and cell extracts were incubated with the anti-CRD antibody. The resulting immune complexes were treated with protein A Sepharose (Pharmacia-Biotech) slurry and the beads were pelleted by centrifugation, and were analyzed by SDS-PAGE under reducing conditions followed by autoradiography.

RESULTS

Binding Affinity of r150 for TGF-β isoforms:

The present inventors have previously reported that in addition to the types I, II and III receptors, keratinocytes express a novel GPI-anchored TGF-β1 binding protein r150 which forms a heteromeric complex with the TGF-β signaling receptors (Tam et al, 1998). Since this protein has the potential to regulate TGF-β signaling, it was further characterized. Here was determined the relative affinity of r150 for the three TGF-β isoforms and, this affinity for TGF-β1 approximates that of the TGF-β signaling receptors, which suggests that r150 is predominantly a TGF-β1 binding protein. Keratinocytes affinity labeled with ^{125}I-TGF-β1 in the absence or presence of increasing concentrations of unlabeled TGF-β1, -β2 or -β3, were analyzed by SDS-PAGE and autoradiography (Figure 1a), and competition curves were created from the autoradiogram for r150, type I and type II receptors using quantitative densitometry (Figures 1b, c, and d). r150 is not sensitive to reducing agents since its migration pattern is identical when the SDS-PAGE analysis is done under non-reducing (Figure 1a) or reducing conditions (Tam et al, 1998). The half-maximal inhibition of ^{125}I-TGF-β1 binding was determined from the competition curves as the TGF-β isoform concentration at which the inhibition was 50% of that observed when no unlabeled ligand was present (Table 1). The concentration of unlabeled TGF-β1 required for half maximal inhibition of ^{125}I-TGF-β1 binding by r150 is only 1.2 and 1.3 times higher than that required by type I and II receptors respectively. Although r150 also binds TGF-β3, it does so with a much lower affinity as compared to the types I and II receptors since it requires a six-fold higher concentration of TGF-β3 to reach half-maximal inhibition of ^{125}I-TGF-β1 binding than the types I or II receptors. Unlabeled TGF-β2, even at 40 times excess concentrations minimally inhibited ^{125}I-TGF-β1 binding of r150.

Partitioning of the membrane bound and released r150 in Triton X-114:

In order to ascertain that the membrane bound r150 is hydrophobic as expected of a GPI-anchored protein and that the released r150 behaves as a hydrophilic soluble protein, the temperature dependent phase separation property of the non-ionic detergent Triton X-114 was used. Phase separation using Triton-X 114 results in the partitioning of hydrophilic proteins into

the aqueous detergent poor phase while integral membrane proteins and lipid attach d proteins partition into the det rg nt rich phase. This proc dure has been useful in distinguishing betwe n the amphipathic (membrane bound) and hydrophilic (released from cell surface) forms of GPI-anchored proteins (Hooper, 1992).

Affinity labeled keratinocytes were subjected to Triton X-114 partitioning and the detergent rich phase containing hydrophobic proteins and the detergent poor phase containing the hydrophilic proteins were analyzed by SDS-PAGE. As expected of a GPI-anchored protein, r150 partitioned predominantly into the detergent rich phase, along with the transmembrane type I, II and III receptors (Figure 2A). The partitioning of soluble r150 in Triton X-114 was then tested. When the detergent rich phase containing the membrane bound affinity labeled r150 was left untreated or treated with PIPLC, and the temperature-induced phase separation was repeated, the aqueous phase of the sample treated with PIPLC was enriched in r150 while that of the sample left untreated contained only low amounts of r150 (Figure 2B). These results strongly indicate that the PIPLC-released r150 is indeed hydrophilic. In contrast, the detergent phase of samples left untreated with PIPLC contained the major portion of r150 while the detergent phase of samples treated with PIPLC contained minimal amounts of r150 (data not shown).

The soluble r150 binds TGF-β1

Was next examined whether r150 released from the cell surface is capable of binding TGF-β1.

Data shown in Figure 3a demonstrate that soluble r150 in the supernatant obtained from keratinocytes treated with PIPLC could be affinity labeled with ^{125}I-TGF-β1. This binding was specific since it was markedly reduced when th labeling was done in the presence of unlabeled TGF-β1. unlabeled TGF-β1 did not exhibit any competition for these complexes.

The low molecular weight bands below 97.4 kDa appear to be nonspecific since unlabeled TGF-β1 did not exhibit competition for these complexes in a reproducible manner. The fact that released r150 binds TGF-β1 indicates that

r150 is capable of binding the ligand in the absence of type I, II and III TGF-β receptors or an intact membrane structure. Interestingly, detectable amounts of r150 were observed in th supernatant not treated with PIPLC, which led us to suspect that there might be an endogenous phospholipase capable of releasing r150.

That the soluble r150 can inhibit TGF-β1 binding to TGF-β receptors was demonstrated using a binding assay. As seen in Figure 3b, the supernatant from PIPLC treated keratinocytes competed in a dose dependent fashion for ^{125}I-TGF-β1 as seen by decreased binding to MvLu1 cells. The supernatant from a T-25cm^2 flask treated with PIPLC inhibited binding by 33% ($p<0.005$) and 50% ($p<0.04$) at doses of 1 and 2 respectively (approximately 1 x 10^6 cells, represented as an arbitrary unit of "1" in Figure 3b). The inhibition of binding with the supernatant from cells not treated with PIPLC is consistent with the observation that detectable amounts of r150 is present in this supernatant, alluding to the presence of an endogenous phospholipase capable of releasing r150 (Figure 3a; also see below, Figure 5). This inhibition of ^{125}I-TGF-β1 binding to MvLu1 cells corresponded to 15%, and 31% ($p<0.03$ in both cases), respectively for doses 1 and 2. In the PIPLC treated supernatants, the inhibition of binding at doses 1 and 2 was significantly higher ($p<0.03$ in both cases) than in the untreated supernatants.

To rule out the possibility that the competition observed by PIPLC treated supernatant was due to TGF-β, the supernatant was neutralized with anti-TGF-β1 antibody prior to being used in the binding assay. Neutralization with this antibody had no effect on the inhibition by the r150 enriched supernatant (Figure 3c). In contrast, 100 pM TGF-β1 markedly inhibited ^{125}I-TGF-β1 binding and this binding could be neutralized by anti-TGF-β1 antibody but not by non-immune IgG. Furthermore, using a PAI-luciferase assay no TGF-β was detected in th supernatants of cells untreated or treated with PIPLC (data not shown). Taken together, these results suggest that the released form of r150 is capable of binding to TGF-β1 and modulating ligand binding to TGF-β receptors.

Identification of a GPI-anchor in r150:

Although r150 is sensitiv to PIPLC, it is possible that it is not itself GPI-anchored, but is associated with a protein that is GPI anchored. Also, it is conceivable that it is a complex of two lower molecular weight proteins which became inadvertently cross-linked during the affinity labeling procedure. In order to eliminat these possibilities, Western blot analysis of r150 was performed after its release from the cell membrane using an anti-CRD antibody specific for an epitope which becomes exposed in GPI anchored proteins only upon treatment with PIPLC.

Keratinocytes were treated with PIPLC and the supernatant was purified on a TGF-β1 affinity column. Analysis of fractions by affinity labeling and SDS-PAGE demonstrated that the fraction 21, but not adjacent fraction (represented by fraction 18) contained a 150 kDa TGF-β1 binding protein (Figure 4a). The binding of ^{125}I-TGF-β1 to this protein is specific since it was blocked in the presence of 5 nM TGF-β1. These results confirm that soluble r150 binds TGF-β1.

When the fractions were analyzed by Western blotting with the anti-CRD antibody, it was revealed that fraction 21, but not other fractions contained a protein of relative molecular weight of 150 kDa which was recognized by the anti-CRD antibody. Detection of a 150 kDa protein by the anti-CRD antibody in the absence of chemical cross-linking demonstrates that r150, but not an associated protein, contains a GPI-anchor and, that r150 does not represent two smaller proteins which got inadvertently cross-linked (Figure 4b). Figure 4b also shows that r150 was not detectable in an adjacent fraction (fraction 18).

Evidence to indicate that an endogenous phospholipase C releases r150 in human keratinocytes:

Next, whether the anti-CRD antibody can immunoprecipitate r150 and/or coprecipitate the types I and II TGF-β receptors was tested. During the course of these studies, it was observed that the anti-CRD antibody immunoprecipitated r150 from ^{125}I-TGF-β1 labeled keratinocytes, even in the absence of PIPLC treatment (Figure 5). This result was reproducible using two different anti-CRD antibodies: the Oxford GlycoSystems antibody that is raised against the CRD epitope of variant surface glycoprotein of *Trypanosoma brucei*

(Oxford GlycoSystems), and the antibody specific for the CRD epitope of the porcine membrane dipeptidase (MDP, Broomfield and Hooper, 1993). Since both anti-CRD antibodies are specific for th inosit l 1,2-cyclic monophosphate epitope xpos d only upon PIPLC treatment, recognition by the two antibodies in the absence of PIPLC treatment indicates that an endogenous phospholipase C cleaved r150 to expose this epitope. The anti-CRD antibody not only precipitated the r150, but also coprecipitated the types I and II receptors. Interestingly, the intensities of the types I and II bands were much stronger than that of the r150 itself. The coimmunoprecipitation of the types I and II receptors demonstrates the heteromeric complex formation of r150 with those receptors (Figures 5a, b) which confirms the inventors' previous finding (Tam et al, 1998). The immunoprecipitation with the anti-CRD antibody is specific because the precipitation of labeled complexes is efficiently blocked when the PIPLC hydrolyzed form of MDP which contains the epitope to which the antibody was raised against was included in the reaction (Figure 5b). These complexes are not detected when the cells were affinity labeled with ^{125}I-TGF-β2 because r150 has a much lower affinity for TGF-β2 than for the TGF-β1 and TGF-β3 isoforms in these cells (Figure 5c).

DISCUSSION

The present inventors have shown that a novel 150 kDa TGF-β1 accessory receptor (r150) forms a heteromeric complex with the TGF-β signaling receptors on human keratinocytes (Tam et al. 1998). This accessory receptor was described as GPI-anchored based on its sensitivity to PIPLC. Here it is demonstrated that the GPI-anchor is contained in r150 itself and not on an associated protein and that it binds TGF-β1 with an affinity similar to those of the types I and II TGF-β receptors. In addition, evidence is provided that r150 is released from the cell surface by an endogenous phospholipase C. The most important finding in the present work is that the released (soluble) form of r150 binds TGF-β1 independent of the signaling receptors.

r150 has been characterized as GPI-anchored, based on its sensitivity to phosphatidylinositol specific phospholipase C (PIPLC). In order to prove that the GPI-anchor is present in the r150 itself, it was necessary to rul out other

possibilities, namely: (i) r150 is not itself GPI-anchored, but is tightly associated with a protein that is GPI-anchored. Upon PIPLC treatment, the associated GPI-anchored protein is cleaved which results in the release of both the proteins into the supernatant. This has been shown to be the case for lipoprotein lipase which was initially identified as GPI-anchored protein based on its sensitivity to PIPLC; but it was later found that its PIPLC sensitivity was a result of close association with a GPI-linked heparan sulfate proteoglycan (Chajek-Shaul et al, 1989). (ii) r150 is a noncovalently associated complex of two lower molecular weight proteins whose combined molecular weights equate 150 kDa, of which one component is GPI-anchored. During affinity labeling, the two proteins get inadvertently crosslinked by the chemical crosslinker BS^3, and thus upon analysis by SDS-PAGE, the cross-linked complex is detected at 150 kDa.

By immunoblotting the purified, soluble form of the r150 with the anti-CRD antibody that can specifically recognize the epitope exposed by the cleavage of the GPI-anchor by PIPLC, the above two possibilities were eliminated. Elution from a TGF-β1 affinity column and detection as a 150 kDa protein in the absence of cross-linking, together with its ability to be recognized by the anti-CRD antibody, prove that r150 has a relative molecular weight of 150 kDa and that the GPI-anchor is contained in r150 itself. There are two GPI-anchored proteins expressed in mammalian tissues that have similar molecular weights as r150. These include an isoform of NCAM (140 kDa) (Rosen et al, 1992) and ceruloplasmin (135 kDa) (Patel and David, 1997). However, immunoprecipitation with antibodies specific to these proteins did not immunoprecipitate r150 affinity labeled with ^{125}I-TGF-β1 (data not shown).

The soluble r150 is capable of binding to TGF-β1 as shown by affinity labeling of the released protein, and retention on the TGF-β1 affinity column. This suggests that r150 can bind TGF-β in the absence of types I, II and III receptors or an intact membrane. That soluble r150 has the potential to modulate TGF-β binding to its receptors was demonstrated by its ability to inhibit ^{125}I-TGF-β1 binding to receptors on mink lung cells. Although studies of the inhibition of TGF-β binding to its receptors by r150 used cellular supernatant and not purified r150, this inhibition

is mo t likely due to r150 itself, since th re was no measurable TGF-β and neutralizing anti-TGF-β antibody had no effect on this inhibition. The inhibition obtained by the sup rnatant not treated by PIPLC is likely due to endogenous release of r150 (Figure 5). As expected, exogenous addition of PIPLC resulted in significantly higher inhibition of binding since more r150 will be released. In addition, r150 is the major TGF-β binder released by PIPLC (Figure 2). The other potential binder, α2-macroglobulin is unlikely to be released in sufficient quantity during the one hour incubation.

That soluble r150 binds TGF-β1 is reminiscent of what is observed of the ectodomain of type III receptor which has been shown to be released by a not yet characterized mechanism (Lopez-Casillas et al, 1994; Philip et al, 1999). The soluble r150 may act as an antagonist by preventing the binding of TGF-β to the signaling receptors as has been suggested for the soluble type III receptor (Lopez-Casillas et al, 1994). This is supported by our finding which suggests that the solubl r150 inhibits ^{126}I-TGF-β1 binding to receptors on mink lung cells. But unlike the typ III receptor, r150 would antagonize TGF-β1 activity in an isoform specific manner, since it has a low affinity for TGF-β2 and a moderate affinity for TGF-β3, as determined by competition experiments using unlabeled TGF-β isoforms. Furthermore, affinity cross-link labeling of keratinocytes with ^{125}I-TGF-β2 or ^{125}I-TGF-β3 did not demonstrate labeling of r150 (data not shown). Recently, a soluble type I receptor has been cloned from a rat kidney cDNA library (Choi, 1999). In contrast to the soluble type III receptor, the soluble type I receptor requires the co-expression of the type II receptor in order to bind TGF-β. However, it appeared to potentiate TGF-β signal transduction and the author has suggested that this potentiation may be due to the stabilization of the heteromeric TGF-β signaling receptor complex. The observation that the soluble r150 can bind the ligand independently of signaling receptors, and modulate TGF-β binding to its receptors (present work), together with fact that the membrane bound r150 binds TGF-β1 and forms heteromeric complex with the type I and II receptors (Tam et al, 1998) raise the possibility that r150 in its membrane bound or soluble form may act as antagonist of TGF-β signaling by regulating ligand availability, or stability of the signaling receptor complex or by directly affecting the signal transduction process.

It is predict d that r150 has dual roles in TGF-β signaling depending on whether it exists as a cell surface anchored protein or as the soluble form. Consequently, a potential mechanism for the regulation of its action would be the hydrolysis of the GPI-anchor. Both the release of the protein from the cell surface and the ability of the soluble form to sequester TGF-β may modulate TGF-β receptor function. Such a possibility *in vivo* is supported by our observation that an endogenous phospholipase C releases r150 from the cell surface. Since the anti-CRD antibody only recognizes GPI-proteins released from the cell surface by PIPLC, and the cells were not pretreated with PIPLC, the results indicate that there is an endogenous phospholipase C in keratinocytes capable of hydrolyzing r150 at the same site as the PIPLC. The soluble r150 identified in the cellular extract is not due to protease activity since proteolytic cleavage will result in a protein of lower molecular weight. Neither can it be due to failure of cells to add the GPI anchor during synthesis, since the antibody will not recognize that protein. It is of interest to note here that r150 was detectable upon overexposure of films in the PIPLC untreated aqueous fractions obtained by Triton X-114 partitioning (data not shown).

Although the presence of a mammalian PIPLC has not been definitively established, the activity of PIPLC-like enzymes have been implicated in the insulin signaling pathway in rat liver (Satiel, 1996). Furthermore, Movahedi and Hooper (1997) have demonstrated that the insulin stimulated release of GPI-anchored proteins from differentiated 3T3-L1 adipocytes occurs via the action of an endogenous phospholipase C. We choose the general term of phospholipase C for the enzyme that releases the r150 in keratinocytes, since the identity and specificity of the enzyme is not confirmed. Nevertheless, our results suggest that the releas of r150 involves an enzyme that hydrolyzes r150 at the same site as PIPLC. PIPLD hydrolyze the GPI-anchor at a different site from PIPLC which does not result in the formation of the inositol 1,2-cyclic monophosphate, and therefore the anti-CRD antibodies that we used in this study cannot recognize PIPLD cleaved proteins (Broomfield and Hooper, 1993). However, PIPLD is expressed in mammalian serum and has been shown to be present in keratinocytes (Xie et al, 1993). Lin et al. (2002), who describe a cell suface antigen named CD109, which appears to be very similar to r150, propose that some GPI anchors are acylat d on inositol, because of CD109

sensitivity to phospholipidase D. It is possible that the activity of both enzymes may be involved in regulating the cell surfac expression of r150 on human k ratinocytes.

GPI-anchored proteins have been reported to bind TGF-β on certain cell lines (Cheifetz and Massague, 1991). More recently, the present inventors reported the presence of GPI-anchored TGF-β binding proteins on early passage human endometrial stromal cells (Dumont et al, 1995) and human skin fibroblasts (Tam and Philip, 1998). Both endometrial stromal cells and skin fibroblasts displayed a 180 kDa GPI-anchored TGF-β1 binding protein and a 65 kDa TGF-β2 binding protein. However, whether the GPI anchor is present in the proteins themselves has not been ascertained for any of them, and the identities of these proteins remain unknown. Interestingly, GPI-anchored proteins have been implicated in the maintenance of the epidermis. When the expression of GPI-anchored proteins was abrogated in the skin by the tissue specific deletion of the *PIG-A* gene, a gene essential for GPI-protein synthesis, mutants died shortly after birth and their skin was wrinkled and scaly in comparison to that of the wild type (Tarutani et al, 1997). Since TGF-β has an important role in epidermal homeostasis, it is conceivable that the GPI-function which is compromised in these mutants is related to dysregulated TGF-β action due to the loss of r150.

Our results from the competition experiments demonstrating that r150 has high affinity for the TGF-β1 isoform suggest that it is an endogenous ligand for this protein. Whether the membrane bound or soluble form of r150 acts as scavenger receptors regulating ligand availability, whether they participate directly in the modulation of downstream signaling, or if the release of the soluble form is a regulated event *in vivo*, remain to be determined. Identification of its structure should facilitate resolution of these issues. Elucidating the mechanism by which r150 functions as an accessory molecule in TGF-β signaling in keratinocytes may be critical to understanding the molecular mechanisms underlying the regulation of TGF-β action.

Example 2

CREATION OF KERATYNOCYTE CELL LINE MUTANTS DEFICIENT IN GPI ANCHOR BIOSYNTHESIS

MATERIALS AND METHODS

Cell Culture: An immortalized human keratinocyte cell line, HaCat, was obtained from Dr. P. Boukamp (Hedielberg, Germany). HaCat is a spontaneously immortalized cell line which displays no major differences in differentiation as compared to normal keratinocytes. It possesses a transformed phenotype, but is not tumourigenic (Boukamp et al, 1988). HaCat cells were cultured in D-MEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine and 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (all Gibco, Burlington, Ontario).

Doubling time: 8 x10^6 cells were seeded at in 60 mm dishes (Falcon) in duplicate. At indicated times, cells were trypsinized and counted using a hemocytometer

Affinity labeling of cells: Affinity cross-link labeling techniques were performed as detailed by Dumont et al (1995). Briefly, monolayers of cells were washed with ice cold binding buffer [PBS with Ca2+ and Mg2+, pH 7.4 (D-PBS) containing 0.1% BSA] three times over a thirty minute period. Cells were then incubated at 4°C for three hours with 100 pM of ^{125}I-TGF-ß1 in the presence or absence of excess non-radioactive TGF-ß1 (Austral Biochemical, Genzyme Inc. or R & D Systems respectively). The receptor-ligand complexes were then cross-linked with 1mM Bis(Sulfocsuccinimidyl)suberate (BS3) (Pierce, Rockford Il). After 10 minutes, th reaction was stopped by the addition of 500 mM glycine and further incubated for 5 minutes. The cells were then washed twice with D-PBS and lysed with solubilization buffer (20 mM Tris-HCl, pH 7.4 containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 µM phenylmethylsulfonylfloride (PMSF), 200 µg/ml BSA, 1 µg/mL leupeptin, 10 µg/ml soyabean trypsin inhibitor, 10 µg/ml benzamide and 2 µg/ml pepstatin). The solubilized material was collected and 1/5 volume of 5X electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol and trace bromophenol blue) was added. The samples were run on a 1.5 mm-thick 3%-

11% SDS-PAGE under nonreducing or reducing (in the presence of 5% ß-mercaptoethanol) conditions. Results were analyzed using autoradiography followed by quantitative densitometry. [^{14}C] methylated molecular weight protein markers included myosin (H-chain) (200-220 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa), ß-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) (Gibco or Amersham-Pharmacia Biotech).

Affinity labeling of soluble r150. Confluent monolayers of keratinocytes were treated with 0.6 U/ml of PIPLC for one hour at 37°C with mild agitation. The supernatant containing the released GPI-anchored proteins was collected and concentrated by Centricon 30 (Amicon). To perform affinity labeling of the soluble form of r150, aliquots of the concentrated supernatant were affinity cross-link labeled with 100-150 pM of ^{125}I-TGF-ß1 in the absence or presence of excess unlabeled TGF- ß1 as described above except that the solubilization step was omitted. Binding complexes were analyzed by SDS-PAGE and autoradiography.

Isolation and cloning of a keratinocyte cell line mutated in GPI anchor biosynthesis. The preparation of a keratinocyte cell line mutated in GPI anchor biosynthesis was performed as described by Stevens (1999). HaCat cells grown to 50-60% confluence in a T-75cm2 flask were treated with 300 µg/ml of ethylmethane sulfonate (EMS) for 24 hours at 37° C in an atmosphere of 5% CO_2/air. EMS is one of the most common chemical mutagens used to generate cells that are defective in GPI anchor biosynthesis (Sega, 1984). Cells were then allowed to recover for an additional 48 hrs. in normal growth medium. Fluorescence activated cell sorting (FACS) was performed to select those cells which lost the expression of GPI-anchored proteins from their cell surface. Since the identity of the r150 was not known, GPI anchor deficient cells were negatively sorted for another GPI-anchored protein, CD59. Briefly, cells were trypsinized and approximately 2 x 10^6 cells were resuspended in one milliliter of FACS buffer (PBS containing 1% FBS) and incubated with 4 µg of fluorescein isothiocyanate (FITC)-conjugated mouse anti- human CD59 monoclonal antibody (BD Pharmagin, Mississauga, ON.) or mouse FITC-conjugated IgG_{2a} monoclonal antibody for 45 minutes on ice. After the incubation, the cell pellet

was washed once and resuspended in one milliliter of FACS buffer. Propidium iodide (3 µg/ml) (Sigma) was added to the cells prior to sorting which was performed using the FACS Vantage (Becton Dickinson). Cell sorting was repeated two additional times. At the third and final FACS, a single CD59 negative cell was seeded into each well of a 96 well plate using the FACS Vantage automated cell deposition unit. In the surviving clones, the cell surface expression of CD59 was analyzed by flow cytometry to determine which cells expressed deficient levels of GPI-anchored proteins. Affinity cross-link labeling with ^{125}I-TGF-ß1 was performed to characterize the expression of the r150.

Luciferase assay. The p3TP-Lux and CMVß-galactosidase (CMVß-gal) gene constructs were gifts from Dr. O'Connor-McCourt (Biotechnology Research Institute, Montreal, Quebec). The p3TP-Lux construct contains three tetradecanoyl phorbol acetate (TPA)-responsive elements and TGF-ß responsive elements from the PAI-1 promoter fused to the luciferase reporter gene (Wrana et al, 1992). Briefly, 2.5 x 10^5 HaCat cells, plated in a 12 well plate , were transiently transfected with 1 µg each of p3TP-Lux and CMVß-gal cDNAs using the Superfect reagent as recommended by the manufacturers (Qiagen, Mississauga, Ontario). The cells were allowed to recover overnight and were serum starved for three to four hours prior to treatment with various doses of TGF-ß1 or TGF-ß2 for the indicated times (Austral Biochemicals). For the luciferase assay, cells were solubilized in 150 µl of lysis buffer (BD Pharmagin, Mississauga, Ontario) for 30 minutes at 4°C. In an opaque 96 well plate, 45 µl of lysate was added to 10 µl of ATP cocktail [0.1 M ATP, 0.5 M KH_2PO_4 (pH 7.8), 1 M $MgCl_2$]. Luciferase activity was measured upon addition of 100 µl of 25 mM luciferin (Roche Dagnostics) in 0.1 M KH_2PO_4 (pH 7.8) using an EG & G Berthold Microplate Luminometer . For the ß-galactosidase assay, in a 96 well plate, 5 µl of the lysate was added to 100 µl of ß-gal buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 M $MgCl_2$ and 50 mM ß-mercaptoethanol) containing 1.5 ng/ml of ortho-nitrophenyl ß D-galactopyranoside (ONPG) (Sigma). The samples were incubated at 37°C until a satisfactory colour reaction was obtained. The colour reaction was then measured at 420 nm using a spectrophotometric plate reader (Molecular Dynamics, Sunnyvale, CA) . Values were derived from transfections performed in duplicate or triplicat and all experiments were performed at least three tim s.

***In vitro* kinas assay.** HaCat and GPI anchor mutated cells grown in T-75cm2 flasks were serum starved overnight and l ft untreated or treated with 100 pM TGF-ß1 for 20 minutes at 37°C. Th cells were scraped in 200 µl of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 50mM NaF, 50 mM ß-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 5 mM EDTA pH 8.0, 1 % NP-40, 10 % glycerol, 10 µM PMSF, 200 µg/ml BSA, 1 µg/mL leupeptin, 10 µg/ml soyabean trypsin inhibitor, 10 µg/ml benzamide and 2 µg/ml pepstatin) and further incubated in the lysis buffer for 30 minutes on ice. The cells were pelleted and the solubilized extract was precleared with 40 µl of protein A Sepharose slurry (Amersham-Pharmacia-Biotech) and 3 µg/ml of polyclonal rabbit IgG antibody for two hours at 4°C. One mg of protein was incubated with 3 µg/ml of anti -type II TGF-ß receptor antibody (Santa Cruz) overnight at 4°C. The immune complexes were then incubated with 60 µl of protein A Sepharose slurry for two hours at 4°C. The beads were pelleted and washed three times with 0.5 ml of lysis buffer and one time with 0.5 ml of kinase buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM $CaCl_2$]. After the last wash, 20 µl of kinase buffer was added to the pellets and incubated with 10 µCi of gamma^{32}P-ATP (NEN) for 30 minutes at 30°C. The reaction was halted by the addition of 5 µl of 5X sample buffer (0.25M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol and trace bromophenol blue) containing 5% ß-mercaptoethanol and boiled for 5 minutes. The extracts were separated on a 7.5% polyacryalamide SDS-PAGE gel and analyzed by autoradiography.

Western blotting of phosphorylated Smad2. HaCat and GPI anchor mutated cells were grown in 60 mm dishes (Falcon) to 70-80% confluency and serum starved overnight. Cells were washed with PBS and were treated with with various doses of TGF-ß1 or TGF-ß2 for the indicated times at 37°C. The cells were solubilized with 500 µl of lysis buffer for at least 30 minutes at 4°C with mild agitation. The cell lysates were collected and centrifuged for 10 minutes at 12 000 x g. The protein concentration of the each sample was normalized to 50 µg using the BioRad protein assay kit as recommended by the manufacturers. 1/5 volume of 5X electrophoresis reducing sample buffer was added to the sample and boiled for 5 minutes. The samples were separated on a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked for at least three hours to

overnight in blocking buffer [TBS-T buffer: 30 mM Tris (pH 7.5), 150 mM NaCl , 0.05% w/v Tw n 20 , containing 5% w/v of skim milk powder]. The blot was then incubated for 60 minutes at room temperature with th PS2 antibody (1:2000 in TBS-T buffer) which recognizes the phosphorylated C-terminus of Smad2 (a gift from Dr. S. Souchelnytskyi, Uppsula, Sweden). The blot was then washed in TBS-T buffer and incubated for 45 minutes at room temperature with a secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (1:5000) (Pierce, Rockford, IL.). The membrane was then subjected to chemiluminescence analysis (ECL) as detailed by the manufacturers (Amersham-Pharmacia-Biotech). In order to determine equal protein loading, membranes were immunoblotted with a goat polyclonal anti-Smad2 antibody (Santa Cruz) that is raised against a peptide near the N-terminus, or a rabbit polyclonal anti-STAT3 antibody (Santa Cruz) which recognizes a peptide mapping at the C-terminus.

RESULTS

Isolation and cloning of a cell line mutated in GPI anchor biosynthesis:

HaCat cells were treated with 300 µg/ml of ethylmethanesulfonate (EMS) for 24 hours as described in "Materials and Methods.". EMS is an ethylating agent that has been used to cause mutagenesis of genes, including those involved in the biosynthesis of the GPI anchor (Sega, 1984; Stevens, 1999). Because the identity of the r150 is not known, another GPI-anchored protein that is strongly expressed in human keratinocytes was used in the cloning of GPI anchor deficient cells. CD59 is a 21 kDa GPI-anchored protein that is expressed in keratinocytes and is an inhibitor of the membrane attack complex (Venneker et al, 1994; Pasch et al, 1998). After EMS treatment, cells were stained with an anti-CD59 antibody conjugated to FITC and negatively sorted. Cells selected into the CD59 negative population were cloned using the FACS Vantage automated cell deposition unit. Ten clones survived and were reanalyzed for CD59 expression by flow cytometry. Two clones displayed decreased expression of CD59 on their cell surface and were presumed to be mutated in GPI anchor biosynthesis (termed GPI M and GPI M1). Figure 6A demonstrates the flow cytometric analysis of parental HaCat cells as compared to GPI M cells. Though the expression of CD59 was not

completely aboli hed in the GPI M cells, th immunofluor scence intensity of CD59 was analyzed to be approximately half that of HaCat cells (56.3% +/- 7.4, p<0.0006). Similar results w re obtained with the GPI M1 cells.

In order to ascertain that the GPI anchor mutated cells ar deficient in r150, affinity labeling with ^{125}I-TGF-ß1 was performed . As demonstrated in Figure 6B, GPI M cells exhibit a significant loss of r150 on their cell surface as compared to HaCat cells. The expression of the types I, II and III receptors and their binding to ^{125}I-TGF-ß1 are unaffected in the GPI M cells and appear identical to that of parental HaCat cells.

Cell growth and morphology are unchanged in GPI anchor mutated cells:

A deficiency or loss of the cell surface expression of GPI-anchored proteins as a result of EMS mutagenesis is reported not to affect cell morphology or cell cycle progression (Stevens, 1999). In order to confirm that cell growth was not altered in the GPI anchor deficient keratinocyte cell line, the doubling time of these cells was determined. As seen in Figure 7A, GPI M cells display a similar doubling time (approximately 16hrs.) as the parental HaCat cells. In addition, the cellular morphology of GPI M cells (Figure 7C) is unchanged to that of the HaCat (Figure 7B).

r150 negatively modulates TGF-ß stimulated transcription:

TGF-ß1 induced transactivation of the p3TP-Lux reporter construct in GPI M and HaCat cells was then evaluated. As demonstrated in Figure 8A, upon treatment with 10 pM TGF-ß1 for 4 hours, the GPI M cells display an approximately 30% higher fold increase of luciferase activity, as compared to HaCat cells ($p<0.05$). In response to 100 pM TGF-ß1, the difference between the GPI M and HaCat cells rises to 50% ($p<0.003$). Interestingly, the fold induction in the HaCat cells with 10 pM and 100 pM TGF-ß1 treatment remain similar to each other (3.3 and 3.9 respectively),while the GPI M cells display a dose response,demonstrating fold increases of 4.9 and 8.4 respectively.

Upon 16 hours of TGF-ß1 treatment, the GPI M cell display a markedly enhanced response to 10 pM and 100 pM TGF-ß1 compared to the parental HaCat (Figure 3B). Also, a dose response to the 10 pM and 100 pM TGF-ß1 are evident for both cell types. A clone of HaCat cells that was subjected to the same cloning procedure as the GPI M cells, but not mutated in GPI anchor biosynthesis (GPI NM) was included as a control. Upon 10 pM TGF-ß1 treatment, GPI M cells display the highest fold induction of 21.4 compared to the 13.6 and 10.6 increases demonstrated by the HaCat and GPI NM cells ($p<0.005$ for GPI M vs HaCat). Similarly, in response to 100 pM TGF-ß1, the fold induction exhibited by the GPI M cells is approximately twice that of HaCat and GPI NM cells (91.2 compared to 50.2 and 51.3 respectively) ($p<0.01$ for GPI M vs HaCat). In response to 100 pM TGF-ß2, there is no significant difference in the stimulation of luciferase activity among the GPI M, HaCat and GPI NM cells (Figure 8C).

GPI anchor mutated cells display enhanced Smad2 phosphorylation at low doses of TGF-ß1:

In order to determine if the loss of r150 had any affect on the endogenous phosphorylation of Smad2, HaCat and GPI M cells were treated with 1-50 pM of TGF-ß1 for 20 minutes and immunoblotting was performed with an antibody raised against the phosphorylated form of Smad2. As demonstrated in Figure 9A, GPI M cells display an enhanced level of Smad2 phosphorylation as compared to the parental HaCat. In both cell types, Smad2 phosphorylation becomes detectable at 2 pM TGF-ß1 and increases in a dose dependent fashion. However, GPI M cells exhibit an enhanced Smad2 phosphorylation in response to low (2 and 5 pM), intermediate (10 pM) and higher (50 pM) doses of TGF-ß1 in comparison to the HaCat cells. Immunoblotting with an anti-Smad2 antibody that detects both the unphosphorylated and phosphorylated forms of Smad2 not only demonstrates equivalent protein loading, but shows that the enhanced Smad2 phosphorylation is not a result of an increased expression of total Smad2. Equal protein loading of HaCat and GPI M cell lysates is also confirmed upon immunoblotting with an anti-STAT3 antibody. Enhanced Smad2 phosphorylation in the GPI M cells is not evident

in response to TGF-ß2, as HaCat and GPI M cells appear to demonstrate the same weak pattern of TGF-ß2 induced Smad2 phosphorylation (Figure 9B).

Elevated Smad2 phosphorylation is sustained in GPI anchor mutated cells:

Was further examined if the GPI anchor deficient cells exhibit enhanced Smad2 phosphorylation as compared to HaCat cells upon prolonged exposure to TGF-ß1 (Figure 10). In response to 100 pM TGF-ß1 for the indicated times, maximal stimulation of Smad2 phosphorylation in HaCat cells is achieved at 10 minutes and maintained for 180 minutes. In GPI M cells, Smad2 is maximally phosphorylated between 45-90 minutes, followed by a moderate decrease in its phosphorylation at 180 minutes. In comparison to HaCat cells, the GPI M cells display a significantly elevated Smad2 phosphorylation which is evident at 45 minutes and is sustained until 180 minutes. The second GPI anchor mutated clone (GPI M1) also exhibits enhanced Smad2 phosphorylation in response to TGF-ß1 (Figure 10B). In this experiment, the GPI M1 cells display an elevated level of TGF-ß1 induced Smad2 phosphorylation as compared to HaCat cells starting at 20 minutes and is sustained until 180 minutes. On the other hand, Smad2 phosphorylation in GPI NM control cells is similar to that of HaCat cells for all the time periods studied in response to TGF-ß1 (Figure 10C). Immunoblotting with the anti-STAT3 antibody demonstrates equal protein loading of the cell lysates. These results indicate that only the GPI anchor deficient cells (GPI M and GPI M1) exhibit enhanced TGF-ß1 stimulated phosphorylation of endogenous Smad2 as compared to the parental HaCat cells.

Autophosphorylation of type II kinase in HaCat and GPI anchor mutated cells.

Since r150 can interact with the TGF-ß signaling receptors in human keratinocytes, and TGF-ß induced Smad2 phosphorylation is enhanced in GPI anchor deficient cells, we analyzed for alterations in the autophoshorylated state of the constitutively active type II receptor kinase using the *in vitro* kinase assay. As demonstrated in Figure 11, GPI M cells do not display any marked differences in type II receptor kinase phosphorylation as compared to the parental HaCat cells in either the absence or presence of 100 pM TGF-ß1. The moderate decrease in TGF-ßRII

kinase autophosphorylation displayed by the GPI M cells, as compared to HaCat cells, did not occur in a reproducible manner.

DISCUSSION

A keratinocyte cell line mutated in GPI anchor biosynthesis (GPI M) derived by CD59 negative based FACS from HaCat cells treated with EMS (Stevens, 1999) was created and isolated. In comparison to the parental HaCat, these cells demonstrate a significant loss in expression of the novel GPI-anchored TGF-ß1 accessory receptor, r150, from their cell surface. There is no alteration in TGF-ß types I and II receptor expression, nor in their abilities to bind TGF-ß1. In addition, GPI M cells do not show any differences in cellular morphology or doubling time. However, these cells display an enhanced transactivation of the TGF-ß responsive p3TP-Lux reporter gene construct as compared to HaCat cells. Furthermore, GPI M cells display an enhanced Smad2 phosphorylation in response to TGF-ß1 treatment in a time and dose dependent manner. Taken together, the present work indicates that the novel accessory receptor, r150 acts as a negative modulator of TGF-ß action in human keratinocytes.

In the isolated GPI anchor deficient cell lines, the cell surface expression of CD59 is still detectable by flow cytometry, thus indicating that the cells do not display a complete abrogation of GPI-anchored proteins. However, a decrease in immunofluorescence intensity of approximately 50% exhibited by the GPI M cells is comparable to the loss of GPI-anchored proteins from the cell surface after PIPLC treatment (Screaton et al, 2000). In addition, ^{125}I-TGF-ß1 affinity cross-link labeling of GPI M cells demonstrate a significant loss of r150 from their cell surface as compared to HaCat cells. That GPI anchor biosynthesis is not completely abolished in these cells is likely due to the presence of GPI anchor biosynthetic genes that are resistant to EMS mutagenesis (Stevens et al, 1996). The present inventors are the first to report of the isolation of a GPI anchor deficient keratinocyt cell line.

EMS is an ethylating agent that has been used to caus mutagenesis of genes, including those involved in the biosynthesis of the GPI anchor

(Sega, 1984; Stevens, 1999). There is a mutation frequency of approximately one in a million cells upon tr atment of mammalian cells with this mutagen in the dose range of 100-400 µg/ml. Henc , to "knock out" two copies of a g n , th frequency becomes 1 000 000 x 1 000 000 (Boyd and Massague, 1989; V. Stevens, personal communication). At this low frequency, the assumption is made that there is one mutation in each cell that is selected and cloned. For example, using this methodology, Stevens et al (1996) created a Chinese Hamster Ovary (CHO) cell line mutated in GPI anchor biosynthesis. This cell line has been recently used to study the role of GPI-anchored proteins in the development of Alzheimer's disease (Sambamurti et al, 1999) and in the "cross-talk" between caveolae and GPI-enriched lipid microdomains (Abrami et al, 2001). In addition, prior to the cloning of the TGF-ß receptors, EMS mutagenesis was used to create the mutant Mv1Lu cell lines known as "R" and "DR" which do not express the type I and types I and II TGF- ß receptors respectively (Boyd and Massague, 1989; Laiho et al, 1990). These TGF-ß unresponsive R and DR mutant cell lines continue to be used to delineat components of the TGF-ß signaling pathway (Massague, 1998). Therefore, the main drawback of the above model is that the expression of all GPI-anchored proteins may be affected. Thus, it is possible that the loss of other GPI proteins besides r150 may have an impact on TGF-ß signaling. However, none of the presently identified mammalian GPI-anchored proteins can bind TGF-ß or are implicated in TGF-ß signaling (for review, Low, 1989; Turner, 1994). The novel 150 kDa accessory receptor is the strongest candidate because it appears to be the only GPI-anchored protein that binds to TGF-ß1 in keratinocytes. Furthermore, it has the potential to modulate TGF-ß signaling through its interaction with the types I and II TGF-ß receptors. Other GPI-anchored TGF-ß binding proteins such as 180 kDa TGF-ß1 binding protein and two GPI-anchored TGF-ß2 binding proteins at 60 kDa and 140 kDa were identified in certain cell lines including a human osteosarcoma cell line (Cheifetz and Massague, 1991). In human fibroblasts, the present inventors identifi d a 180 kDa GPI-linked TGF-ß1 binding protein, as well as a 65 kDa GPI-anchored TGF-ß2 binding protein which do not interact with the types I and II TGF-ß receptors (Dumont et al, 1995; Tam and Philip, 1998). However, early passage human keratinocytes and HaCat cells do not appear to express any of GPI-anchored TGF-ß

binding complexes with the above mentioned relative molecular weights (Chapter 4; Tam et al, 1998). Together, it is likely that the observed differences in TGF-ß1 induced cellular r spons s xhibit d by the GPI M cells is due to the loss of r150.

The GPI anchor deficient cells display an increased transactivation of the PAI-1 promoter driven luciferase gene construct upon 4 and 16 hours of TGF-ß1 treatment. The fold induction demonstrated by the GPI M cells is approximately twice that of HaCat cells and of an isolated keratinocyte clone that was not characterized as being mutated in GPI anchor biosynthesis (GPI NM). Th conclusion that r150 is a negative modulator of TGF-ß responses is confirmed by enhanced phosphorylation of endogenous Smad2 exhibited by the GPI anchor deficient cells as compared to the parental HaCat. This phenomenon is reproducible in both GPI anchor mutants that were cloned (GPI M and GPI M1). In comparison to the HaCat cells, both GPI M and GPI M1 demonstrate a significantly elevated lev l of Smad2 phosphorylation which is detectable after 20-45 minutes of TGF-ß1 treatment and sustained for as long as 180 minutes. Additionally, the GPI M cells demonstrate an enhanced Smad2 phosphorylation in response to different doses of TGF-ß1 (2-50 pM) as compared to the parental HaCat cells. Upon treatment with TGF-ß2, there is no detectable alteration in the pattern of Smad2 phosphorylation between HaCat and GPI M cells, indicating that the loss of r150 may not impact TGF-ß2 responsiveness. This is also seen in the p3TP-Lux luciferase assay, whereby the HaCat and GPI M cells display similar levels of TGF-ß2 induced luciferase activity. This is not surprising since r150 has virtually no affinity for TGF-ß2 (Tam et al, 1998). The increased intensity and duration of the TGF-ß1 induced Smad2 phosphorylation likely contributes to the enhanced transactivation of the PAI-1 promoter in the GPI anchor deficient cells.

r150's interaction with the types I and II receptors is reminiscent of another accessory receptor, endoglin. Endoglin is a 180 kDa homodimeric transmembrane protein that binds TGF-ß1 and TGF-ß3, and can interact with th types I and II receptor (see section 1.2.2 ; Cheifetz et al, 1992; Yamashita et al, 1994b). Like the r150, it possesses no kinase domain, but endoglin displays a short cytoplasmic region that is constitutively phosphorylated (Yamashita et al, 1994b).

Furthermore, overexpression of endoglin exerts an inhibitory effect on TGF-ß m diated growth inhibition, PAI-1 induction and angiog nesis (Letamendia et al, 1998; Li et al, 2000). However, unlik r150, endoglin cannot bind TGF-ß in the absence of the type II receptor and hence, its interaction with the signaling receptors is a ligand induced phenomenon. Furthermore, endoglin is predominantly expressed in endothelial cells while our studies indicate that r150 is not found in endothelial cell types (Wong et al, 2000; Tam and Philip, unpublished observations). Therefore, it is possible that the mechanisms by which r150 and endoglin exert their inhibitory effects are distinct from each other.

What are the potential molecular mechanisms by which r150 regulates TGF-ß signaling? Through its interaction with the TGF-ß signaling receptors, r150 may modulate the serine/threonine kinase activity of the types I or II receptors. The autophosphorylation of key residues in the type II receptor is important in the regulation of kinase function (Luo and Lodish, 1997). *In vitro* kinase assay with the anti- type II receptor antibody indicates that there is no mark d difference in the type II autophosphorylation in GPI anchor deficient cells as compared to that of HaCat cells in the absence or presence of TGF-ß1 (Figure 11). It is possible that r150 may interact with the type I receptor to hamper its phosphorylation by the type II receptor, and hence, activation of the type I kinase. This would be similar to the action of FKBP12, an immunophilin which interacts with the type I kinase and exerts an inhibitory effect on TGF-ß mediated cellular responses (Wang et al, 1996) . However, our attempts to assess the phosphorylation of the type I kinase by the *in vitro* kinase assay with the anti-type I receptor antibody using previously described methods were unsuccessful (Wrana et al, 1994; Wies r et al, 1995). Alternatively, r150's interaction with the type II receptor may exert a negative regulatory effect on TGF-ß signaling as is seen with TRIP-1, a WD domain protein that specifically associates with the type II receptor and suppresses TGF-ß induced transactivation of the PAI-1 promoter (Choy and Derynck, 1998). However, it is presently unclear if r150 interacts with the signaling receptors in the absence of TGF-ß, or if the interaction is a strictly a ligand induced phenomenon. In addition, it is unknown if r150 preferentially interacts with the type I or type II receptor.

Our previous work indicat s that soluble r150 retain its ability to bind TGF-ß1 in abs nce of the types I and II receptors and of an intact membrane (Tam et al, 2001). Hence, it is likely that r150 binds to TGF-ß1 independently from the TGF-ß signaling receptors when attached to the cell surface. This is in contrast to the type I receptor kinase and endoglin which only recognize TGF-ß bound to the type II receptor (Cheifetz et al, 1992; Wrana et al ,1994; Letamendia, 1998). Therefore, the membrane-anchored form of r150 may regulate TGF-ß1 binding to its receptors. This possibility can be interpreted from the results which show that GPI anchor deficient cells display an enhanced Smad2 phosphorylation even at low and intermediate doses of TGF-ß1 (2-10pM) as compared to the parental HaCat. Furthermore, in the p3TP-Lux luciferase assay, a dose response is demonstrated by the mutant cells upon 4 hours of 10 pM (4.9 fold induction) and 100 pM TGF-ß1 (8.4 fold induction) treatment. In contrast, the fold increases in HaCat cells are similar at both doses (3.3 and 3.9 respectively). Hence, the access of TGF-ß1 to its receptors appears to be ameliorated in the absence of r150. Therefore, unlike the type III receptor whose role is to facilitate binding of TGF-ß to its signaling receptors, r150 may not play the role as an "enhancer" of TGF-ß binding (Lopez-Casillas et al, 1993). As a result, the membrane-anchored r150 may act to sequester TGF-ß1, but not TGF-ß2 or TGF-ß3, away from the signaling receptors on the cell surface. Thus, in the absence of r150, lower amounts of TGF-ß1 can induce the heteromerization of the types I and II receptors which can surpass the signaling threshold required to activate the type I kinase, leading to Smad2 phosphorylation.

r150 may potentially have a role in the cellular "compartmentalization" of the types I and II receptors and of the R-Smads. There is an emerging theme in signal transduction biology whereby signaling molecules can be compartmentalized into membrane entities known as caveolae and "lipid rafts." These are organized lipid microdomains which as serve centres in which signaling molecules of various pathways can effectively interact (Sargiacomo et al, 1993; Horejsi et al, 1999). GPI-anchored proteins have been detected in caveolae or lipid rafts in association with other signaling molecules such as Src-like kinases, G-proteins, PKC, and PDGF receptor (Sargiacomo et al, 1993; Lisanti et al, 1994; Oka et al, 1997; Liu t al, 1997). The association of r150, a GPI-anchored protein, with

th typ I and II receptors suggests that the TGF-ß receptors may also be organized in these plasmalemmal ntities. This is a po sibility since caveolin-1, the main scaffolding protein in caveola , was recently reported to co-localize with th typ s I and II receptors and Smad2 in caveolae-enriched membrane fractions. The type I receptor was demonstrated to directly associate with caveolin-1 which resulted in the downregulation of TGF-ß mediated transcriptional responses and suppression of ligand induced Smad2 phosphorylation (Razani et al, 2001). Furthermore, the FYVE domain in Smad anchor for receptor activation (SARA), a protein that plays a prominent role in controlling the subcellular localization of Smad2, was required to maintain SARA localized in punctate "spots" near the cell surface as characterized by immunofluorescence microscopy (Tsukazaki et al, 1998). SARA was shown to recruit Smad2 into these domains where TGF-ß receptors also co-localized. The identity of these "spots" were not confirmed , but are speculated to be lipid rafts. Presently, it is not known whether SARA interacts with r150. That SARA and r150 may synergistically act to localize r150 into lipid microdomains is an interesting possibility. Within caveolae or lipid rafts, the TGF-ß signaling machinery may also potentially interact with signaling molecules of other pathways that reside in these structures. This would provide a structural explanation as to how TGF-ß can elicit the participation of multiple signaling pathways.

GPI-anchored proteins are implicated in the maintenance of skin homeostasis. Targeted deletion of the GPI anchor biosynthetic gene, PIG-A , in the epidermis of transgenic mice results in smaller pups exhibiting skin with a wrinkled appearance and thickened stratum corneum as compared to wild type mice (Tarutani et al, 1997). This defect in skin development is the apparent cause of death of these mice within 1-3 days after birth. Interestingly, transgenic mice engineered to overexpress TGF-ß1 in the epidermis also demonstrate a compact stratum corneum with an increase in the stratified cell layers as compared to wild type mice (Sellheyer et al, 1993). Due to the overexpression of TGF-ß1, there is a significant reduction in the number of proliferating epidermal cells as determined by pulse labelling with 5-bromodeoxyuridine. Death of these transgenic mice is also attributed to abnormal skin development. Taken together, the targeted overexpression of TGF-ß1 in th pid rmis appears to mimic that of the PIG-A deletion. It is possibl to

envision that the ablation of r150's expr ssion in the epidermis as a consequence of th PIG-A deletion would result in the loss of its inhibitory eff cts in TGF-ß action, thus contributing to the subsequent hyperactivity of TGF-ß1 signaling in the skin. Hence, r150 may play an essential role in skin development as a key regulator of TGF-ß's function in epidermal differentiation and homeostasis.

In conclusion, studies of TGF-ß responses in the GPI anchor deficient keratinocytes have provided critical insight into r150's function in TGF-ß signaling. The present results indicate that r150 negatively modulates TGF-ß action in human keratinocytes. In r150 deficient keratinocytes, TGF-ß induced Smad2 phosphorylation and PAI-1 expression are enhanced . It is conceivable that r150 may directly modulate type I and II kinase activity through its interaction with the signaling receptors. In addition, due to r150's ability to bind TGF-ß1 on its own, the membrane bound and soluble r150 may regulate ligand availability by acting as a scavenger receptor. Delineation of r150's structure is necessary to elucidate the precise molecular mechanisms by which this novel accessory receptor regulates TGF-ß signaling.

EXAMPLE 3

SEQUENCE OF r150 AND NUCLEIC ACIDS ENCODING THE SAME

Cloning of r150 and expression of r150 gene confirm that r150 is an inhibitor of TGF- responses: To determine its structural identity, r150 was purified on a TGF-1 affinity column and analyzed by tandem mass spectrometry (Harvard Microchemistry Facility, Harvard Univ) which allowed us to obtain a 19 amino acid microsequence. This matched to the 5' end of an express sequence tagged (EST) cDNA clone of unknown function from human placenta. Subsequently, sequencing by PCR using seven primers, the full sequence was obtained. Alignment and conserved domain analysis revealed it to be a novel protein of 1428 amino acids with an N-terminal signal sequence and a C-terminal GPI anchor attachment signal sequence (Figure 12 and SEQ ID No: 2). The most likely GPI anchor cleavage site (ω) is at amino acid residue 1404. The predicted molecular mass and structural features indicate that this gene product represents r150. This novel TGF- accessory receptor has a thiolester signature motif and belongs to the complement C3/ 2-

macroglobulin superfamily as recently found in Lin *et al.* (2002). These authors, using a v ry different strategy (affinity binding of blood cells types with monoclonal antibodies) hav identified a protein which has 1445 amino acids, which is very similar to r150. No definite function has been assigned to this protein called CD109, considered by the present inventors to be a variant of r150. Expression studies in HaCaT and 293 cells confirm that the cDNA encodes a 150 kDa GPI- anchored protein.

Transfection of HaCaT and 293 cells with the r150 gene (SEQ ID NO: 1) and detection of the expressed protein by an antibody specific for GPI anchor (anti-CRD antibody) demonstrate that the expressed protein migrates at 150 kDa and that it contains a GPI anchor as expected of r150 (Figure 13). Furthermore, overexpression of r150 in HaCaT and 293 cells results in strong negative modulation of TGF- - induced Smad 2 phosphorylation and gene promoter activity, providing strong evidence to confirm that r150 is an inhibitor of TGF- signaling and responses. Cells transfected with r150 displayed markedly decreased Smad 2 phosphorylation upon stimulation with TGF- when compared with untransfected cells or cells transfected with the empty vector (Figure 14). Similarly, transfection of cells with the 3TPLUX promoter-reporter construct (encoding plasminogen activator inhibitor promoter linked to the luciferase reporter gene) resulted in a marked decrease in both the basal and TGF- induced promoter activity (Figure 15). Taken together, our results demonstrate that r150 is an inhibitor of TGF- signaling and responses *in vitro* and implicate r150, in its membrane anchored and/or soluble form, as a key regulator TGF- action *in vivo* (Figure 16).

Sequence comparisons with the ones disclosed by Lin et al (2002) and Schuh et al. (2002) provide indications of variants (see Figure 17). Some amino acid residues may change, but would presumably not change the property of binding TGF-β1. At least two types of r150-like would exist: one with Tyr, one with Ser at position 703. The TGF-β1 binding region can be predicted by sequence comparison with the sequences disclosed for α_2 macroglobulin (Webb et al. 1998). The TGF-β1 binding domain is ascribed between amino acids 591 and 774 of α_2-M protein sequence. The minimal binding sequence appears to be the 16-mer

WDLVVUNSAGVAEVGU (Webb t al. 2000). r150 corr sponding quence shows little homol gy with this sequence. However, the surrounding sequences are more homologous to r150. Besides that, r150 appears to be much more selective than α_2M for TGF-β1. It is therefore possible that the above 16-mer is sort of a "consensus" sequence with allows versatility in the binding of a plurality of cytokins. The corresponding r150 sequence would be much more specific to TGF-β1. This sequence is SEQ ID No: 12 for r150 and its coding nucleic acid is defined in SEQ ID NO: 11. Any protein comprising this particular minimal 16-mer is within the scop of this invention.

Example 4:

Mapping the TGF-β binding domain of r150

To confirm that r150 binding domain corresponds to th one found for α_2M, the following procedure is performed.

The ligand binding domain of r150 is mapped by producing deletion mutants of r150 and analyzing ligand binding activity. The requirement for the GPI anchor for r150 function is examined using chimeric constructs in which the C-terminal GPI anchor sequence of r150 is replaced with the transmembrane domain (TM) or the GPI anchor sequence of an irrelevant protein, and determining alterations in TGF-β responses. The deletion mutagenesis and creation of chimeric constructs is done as described previously in collaboration with Dr. Uri Saragovi, McGill. (Taheri et al, 2000; Zaccaro et al, 2001)

Deletion mutants are generated using progressive digestion of r150 cDNA using BAL-31 exonuclease starting ~20 amino acids down stream from the signal peptide. Digests are repaired and the fragments are sub-cloned to reattach the signal peptide. The deletion mutants, the full length r150 or the empty vector are expressed in COS-7 cells and in GPI mutant keratinocytes as previously described (Pepin et al, 1994). Cells will then be affinity labeled with 125I-TGF-β 1 and analyzed by SDS-PAGE to determine ligand binding activity as described previously (Tam and Philip, 1998). The generation of chimeric constructs (r150 in which its GPI

anchor sequence is replaced with the TM of insulin receptor or with the GPI anchor sequence of NCAM), was performed using overlapping PCR as previously described (Screaton t al, 2000; Zaccaro t al, 2001). Wild type r150 and the chimeric constructs are expressed in COS-7 cells and in GPI mutant keratinocytes, and TGF-β responses are determined.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. The use of a protein comprising any one of the following sequences in the making of a medication for inhibiting TGF- β1 activity in a biological tissue: SEQ ID Nos: 2 , 4, 6, 8, 10 and 12.

2. The use of an antagonist to a protein comprising any one of the following sequences in the making of a medication for increasing TGF-β1 activity in a biological tissue: SEQ ID Nos: 2 ,4, 6, 8, 10 and 12.

3. The use of a nucleic acid encoding a protein comprising any one of the following sequences in the making of medication for inhibiting TGF-β1 activity in a biological tissue: SEQ ID Nos: 1, 3, 5, 7, 9 and 11.

4. The use of a molecule which silences the expression of a nucleic acid encoding a protein comprising any one of the following sequences in the making of medication for increasing TGF-β1 activity in a biological tissue: SEQ ID Nos: 1, 3, 5, 7, 9 and 11.

5. The use of claim 4 wherein said molecule is an antisense nucleic acid.

6. An isolated nucleic acid encoding a protein comprising any one of the following sequences : SEQ ID Nos: 2,4, 6, 8 and 10.

7. The nucleic acid of claim 6, which comprises any one of the following nucleotide sequences: SEQ ID No: 1 , 3, 5, 7, 9 and 11.

8. The nucleic acid of claim 6, which comprises the nucleotide sequence defined in SEQ ID No: 1, 3, 5 or 7.

9. The nucleic acid of claim 6, 7 or 8, which is a recombinant vector.

10. The nucleic acid of claim 9, which is an expression vector.

11. A recombinant host cell which comprises the nucleic acid of any

one of claims 6 to 10.

12. A method of producing a protein comprising any one of the following sequences : SEQ ID NOs: 2, 4, 6, 8, 10 and 12, which comprises the steps of:

- growing recombinant host cell as defined in claim 11 in a culture medium supporting cell growth and expression of said nucleic acid:
- recovering the protein from the culture medium or from the cell.

13. A nucleic acid which is an antisense nucleic acid of any one of the nucleotide sequences defined in SEQ ID No: 1 or 5.

14. The nucleic acid of claim 13, which is a recombinant vector.

15. The nucleic acid of claim 14, which is an expression vector.

16. A recombinant host cell which comprises the nucleic acid of any one of claims 13, 14 or 15.

17. A TGF-ß1 binding reagent, which comprises a protein comprising any one of the following sequences: SEQ ID Nos: 2, 4, 6, 8, 10 and 12.

18. A composition of matter which comprises the reagent of claim 17, and a carrier.

19. The composition of claim 18, wherein said carrier is a pharmaceutical carrier.

20. The reagent as defined in claim 17, 18 or 19, wherein said protein is hydrosoluble and has the amino acid sequence of SEQ ID No: 4 or 8.

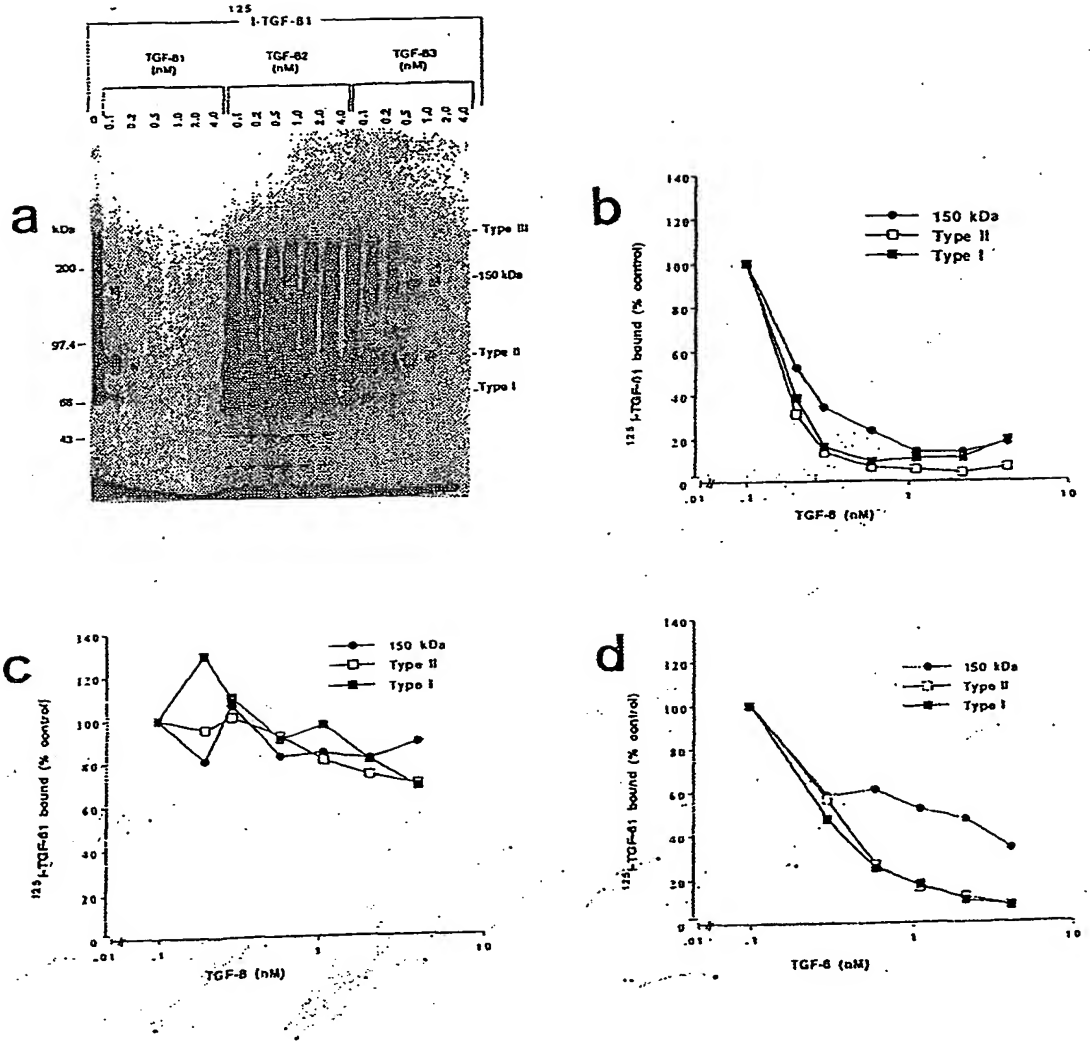

a
125 I-TGF-β1
TGF-β1 (nM)
TGF-β2 (nM)
TGF-β3 (nM)
kDa
200
97.4
68
43
Type III
150 kDa
Type II
Type I
b
150 kDa
Type II
Type I
125 I-TGF-β1 bound (% control)
TGF-β (nM)
c
150 kDa
Type II
Type I
125 I-TGF-β1 bound (% control)
TGF-β (nM)
d
150 kDa
Type II
Type I
125 I-TGF-β1 bound (% control)
TGF-β (nM)

FIGURE 1

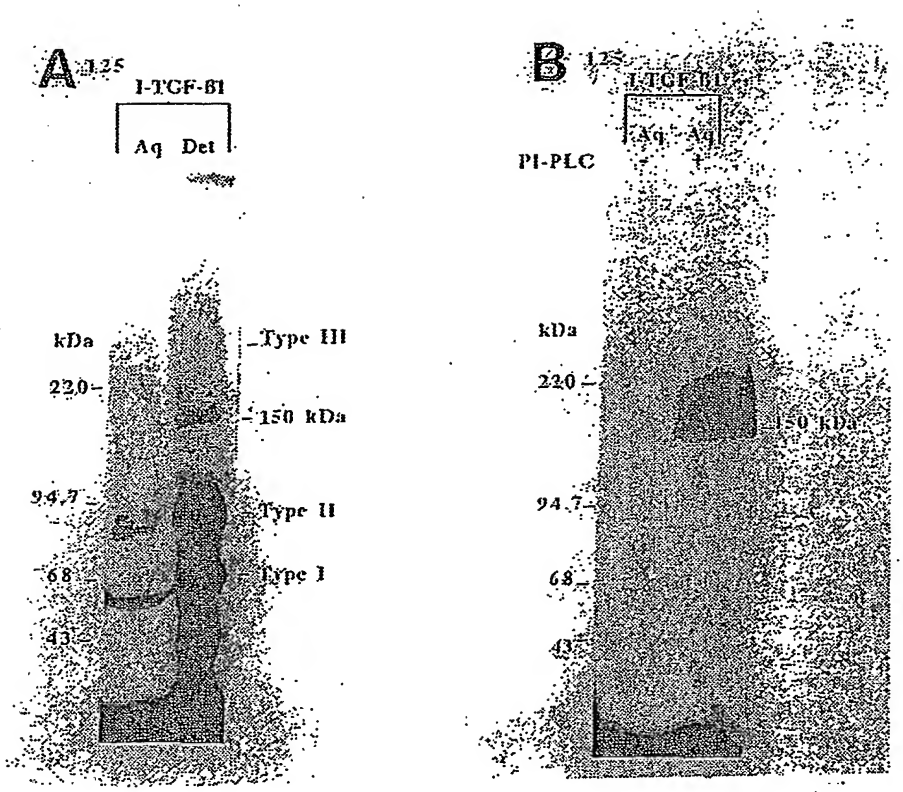

A
125
I-TGF-B1
Aq Det
kDa
220
94.7
68
43
Type III
150 kDa
Type II
Type I
B
125
I-TGF-B1
Aq Aq
PI-PLC
kDa
220
94.7
68
43
150 kDa

FIGURE 2

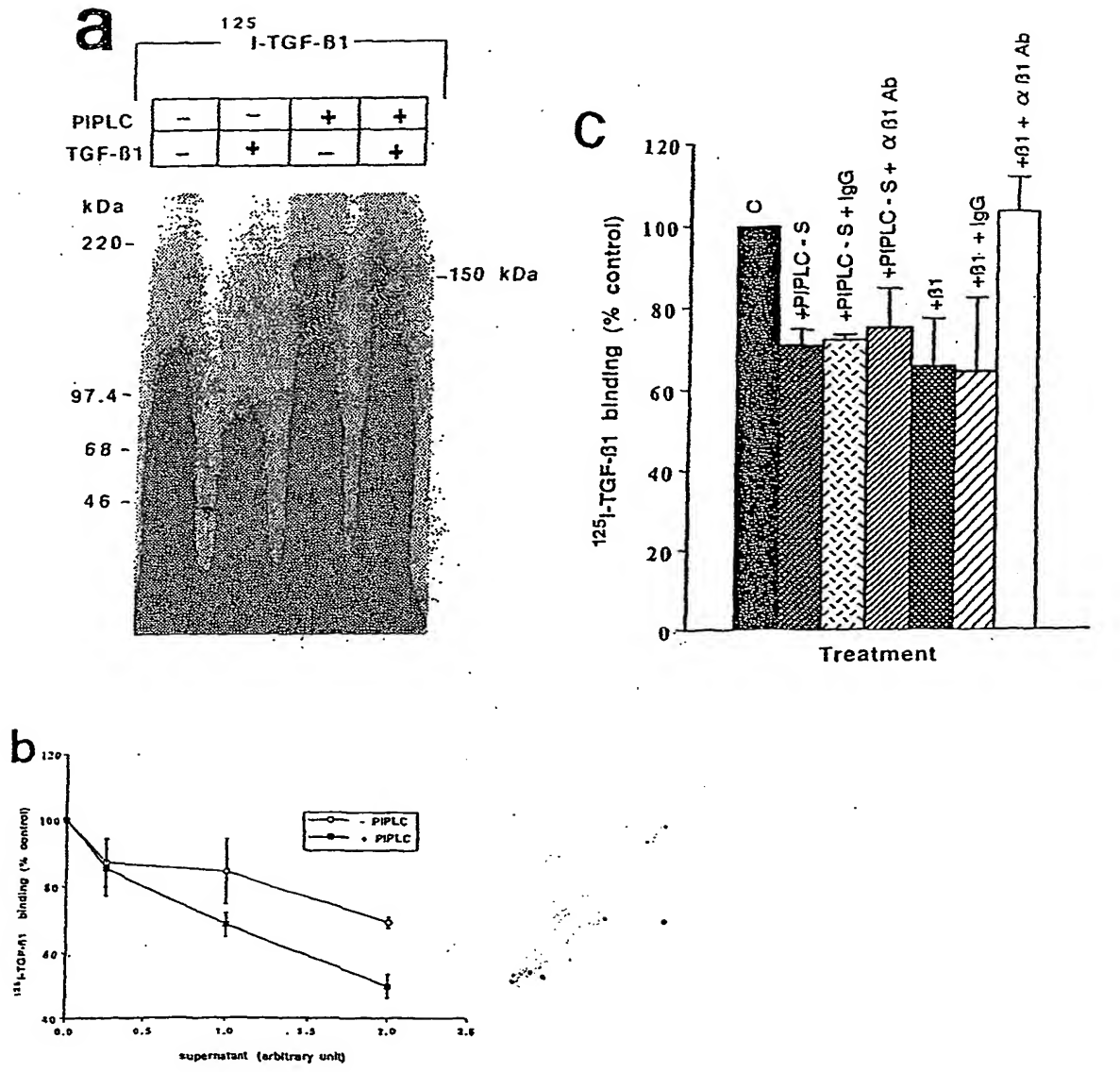

a
125 I-TGF-β1
PIPLC
TGF-β1
kDa
220-
97.4-
68-
46-
150 kDa
c
125I-TGF-β1 binding (% control)
C
+PIPLC - S
+PIPLC - S + IgG
+PIPLC - S + α β1 Ab
+β1
+β1 + IgG
+β1 + α β1 Ab
Treatment
b
- PIPLC
+ PIPLC
supernatant (arbitrary unit)

FIGURE 3

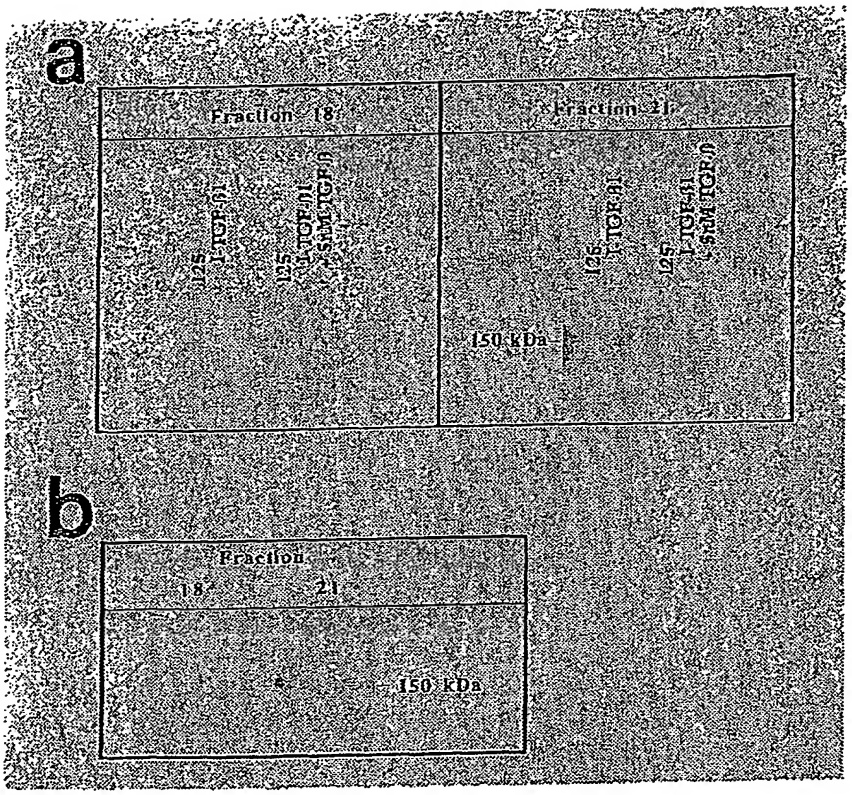


FIGURE 4

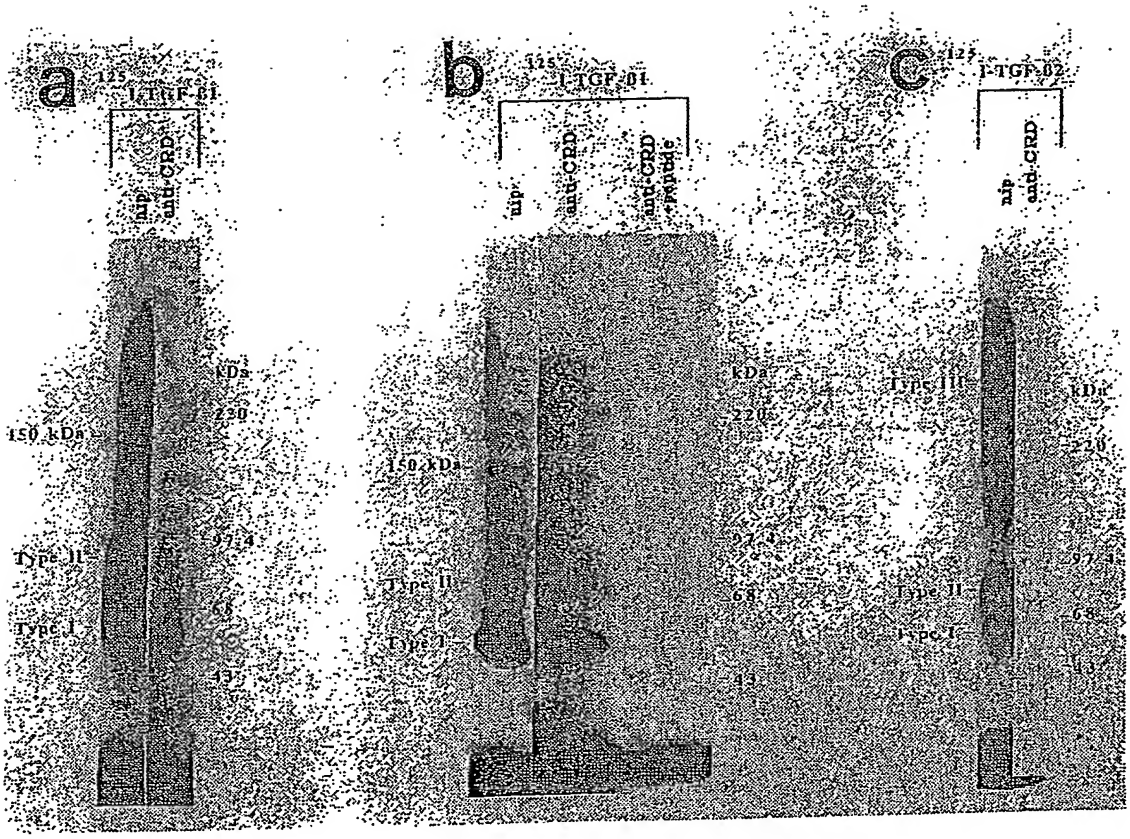

a
125
I-TGF-01
a1
a2
and CRD
kDa
220
150 kDa
97.4
Type II
68
Type I
43
b
125
I-TGF-01
a1
a2
and CRD
and CRD
variable
kDa
220
150 kDa
97.4
Type II
68
Type I
43
c
125
I-TGF-02
a1
a2
and CRD
kDa
Type III
220
97.4
Type II
68
Type I
43

FIGURE 5

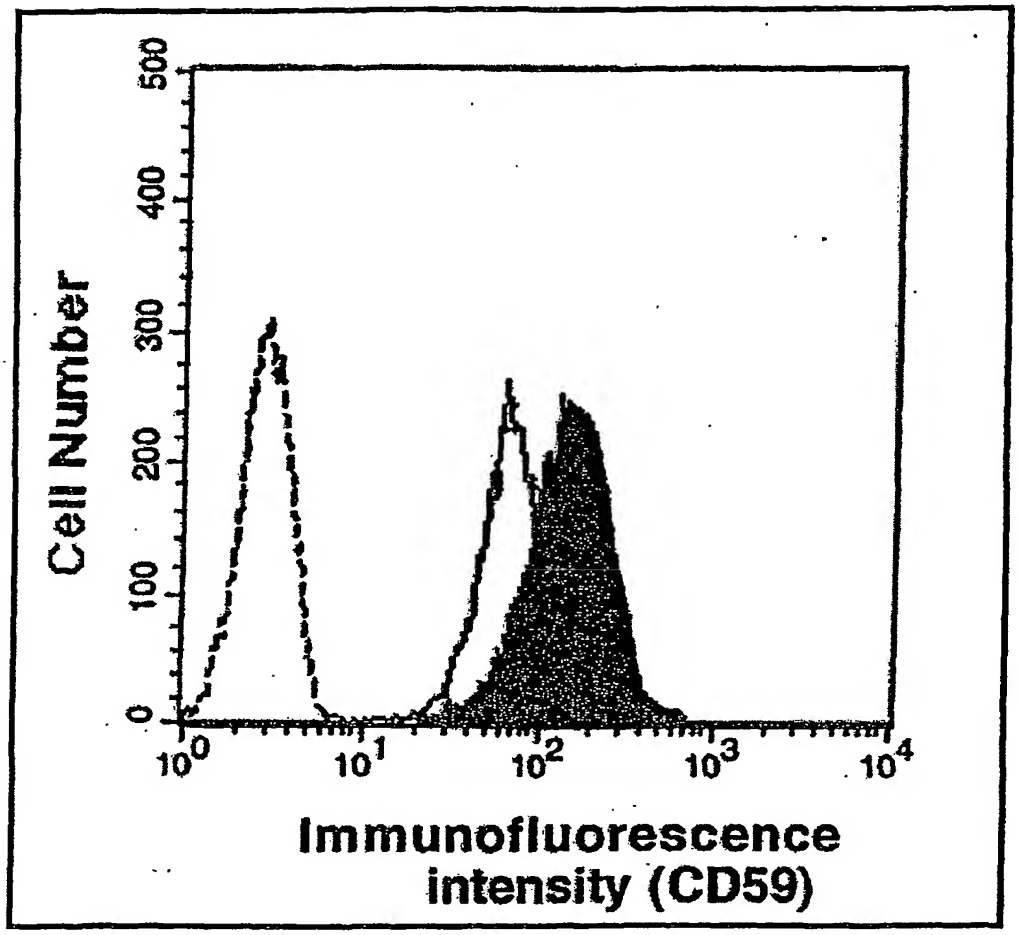

Cell Number
0
100
200
300
400
500
10^0
10^1
10^2
10^3
10^4
Immunofluorescence intensity (CD59)

FIGURE 6 A

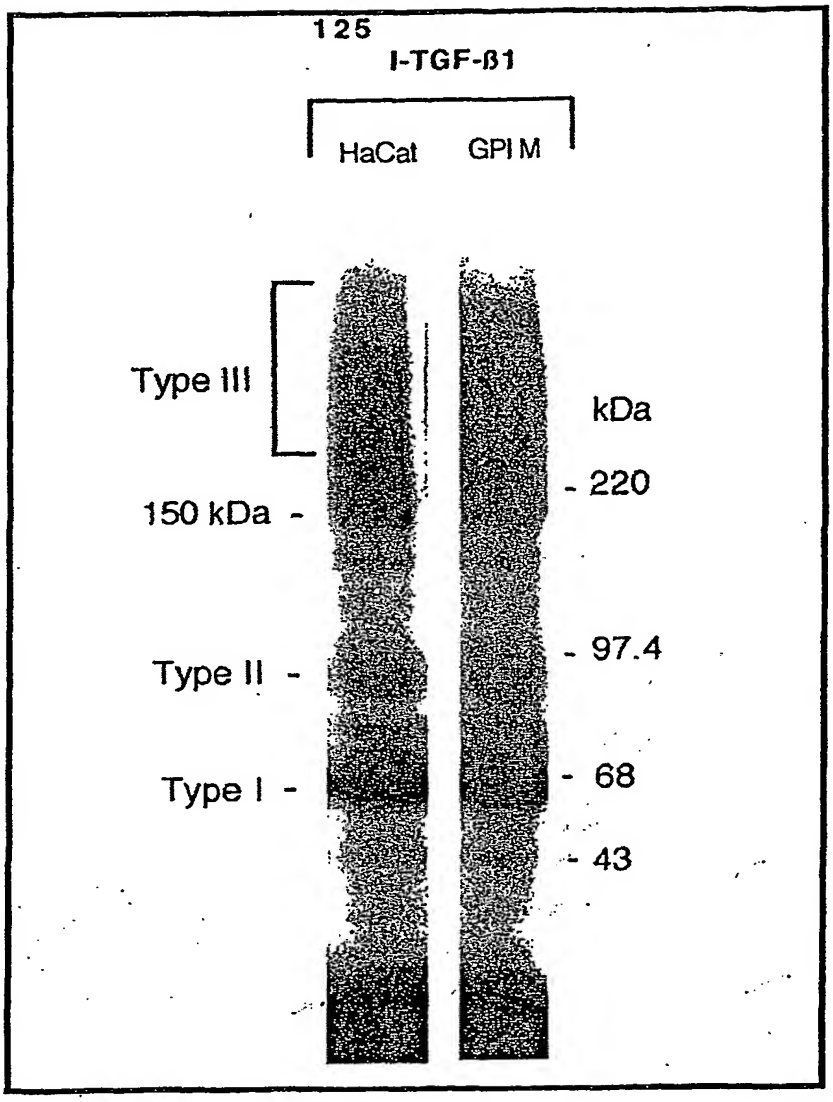

125
I-TGF-ß1
HaCat
GPI M
Type III
150 kDa
Type II
Type I
kDa
220
97.4
68
43

FIGURE 6B

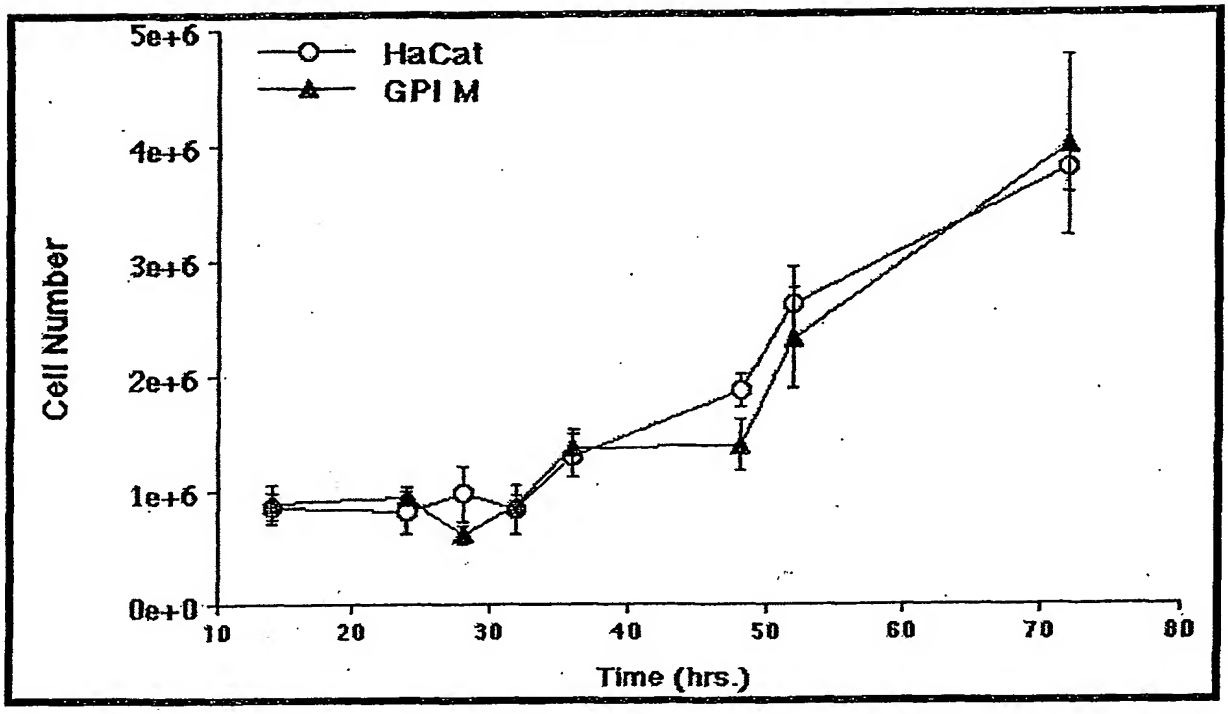

HaCat
GPI M
Cell Number
5e+6
4e+6
3e+6
2e+6
1e+6
0e+0
10
20
30
40
50
60
70
80
Time (hrs.)

FIGURE 7 A

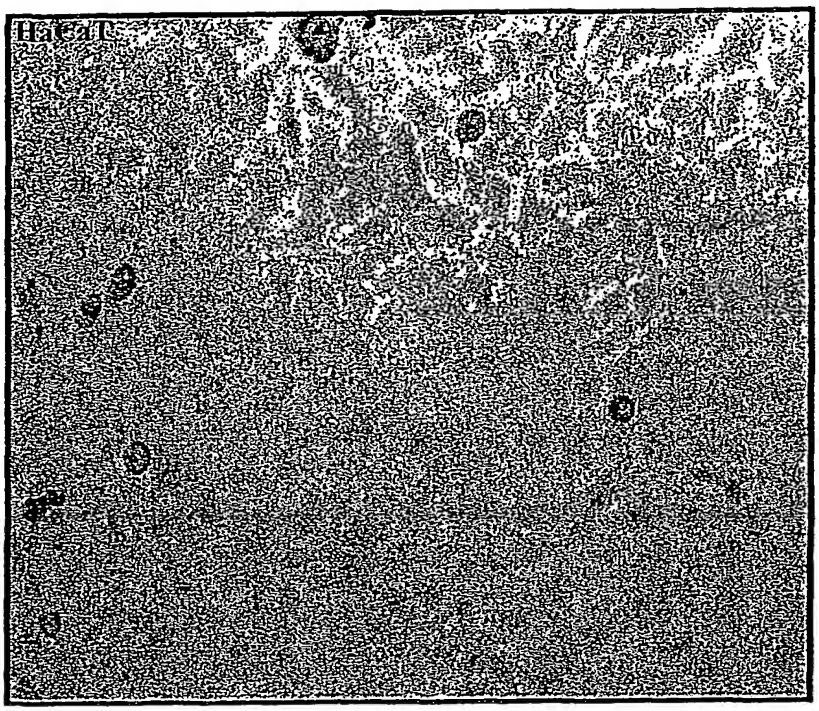


FIGURE 7 B

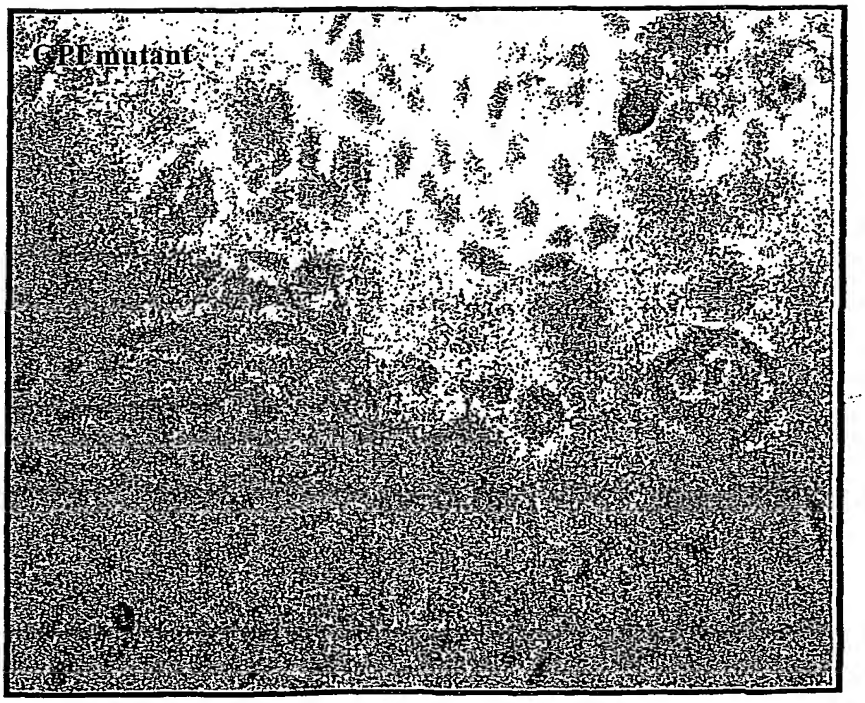


FIGURE 7 C

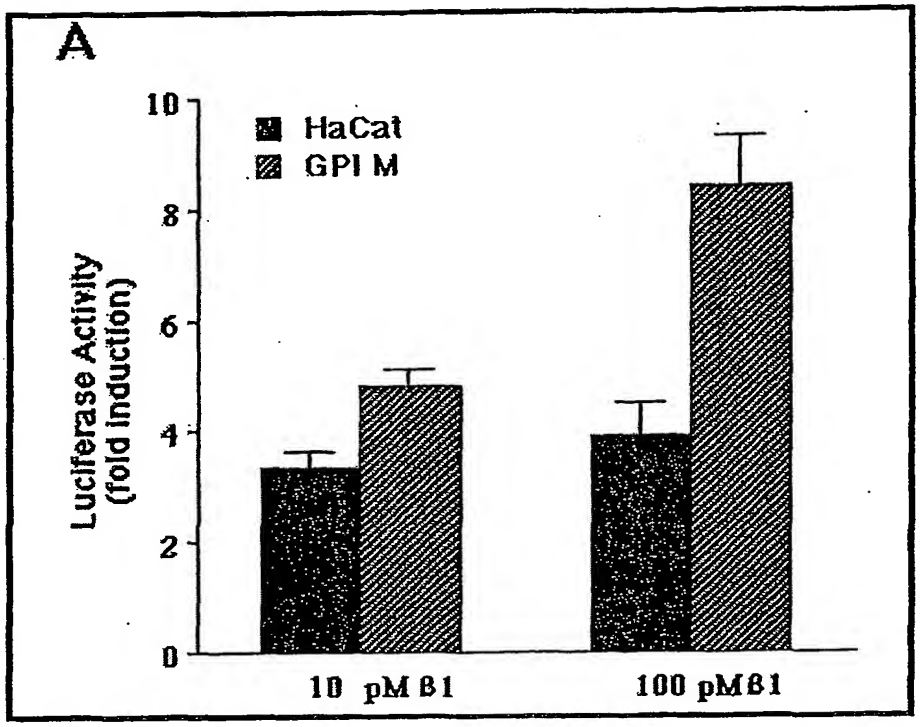


FIGURE 8 A

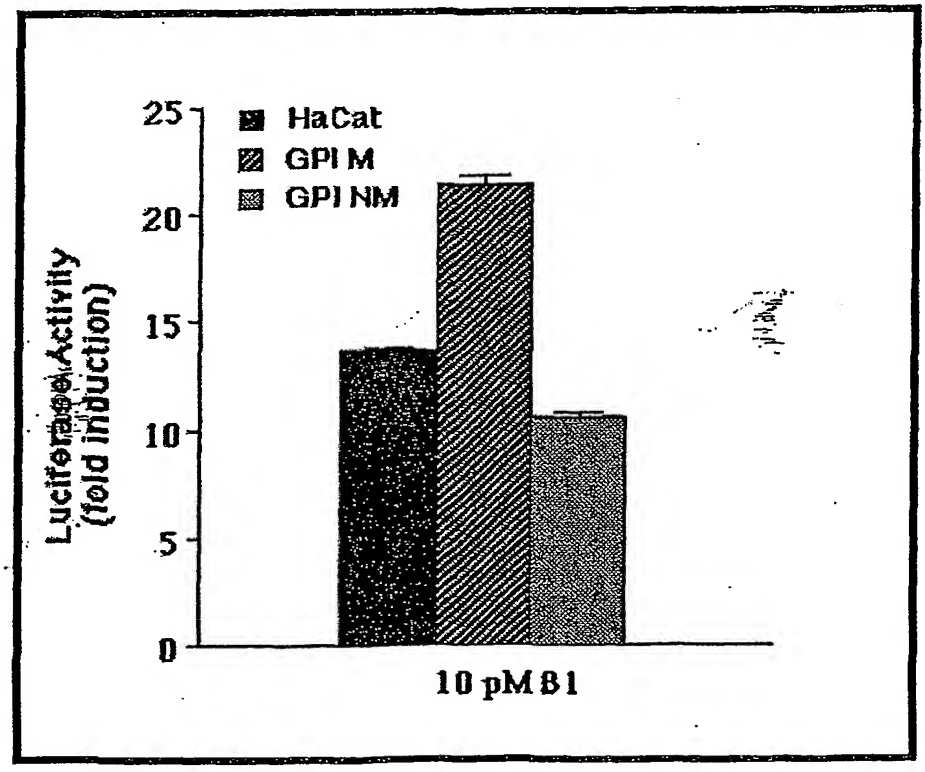


FIGURE 8 B

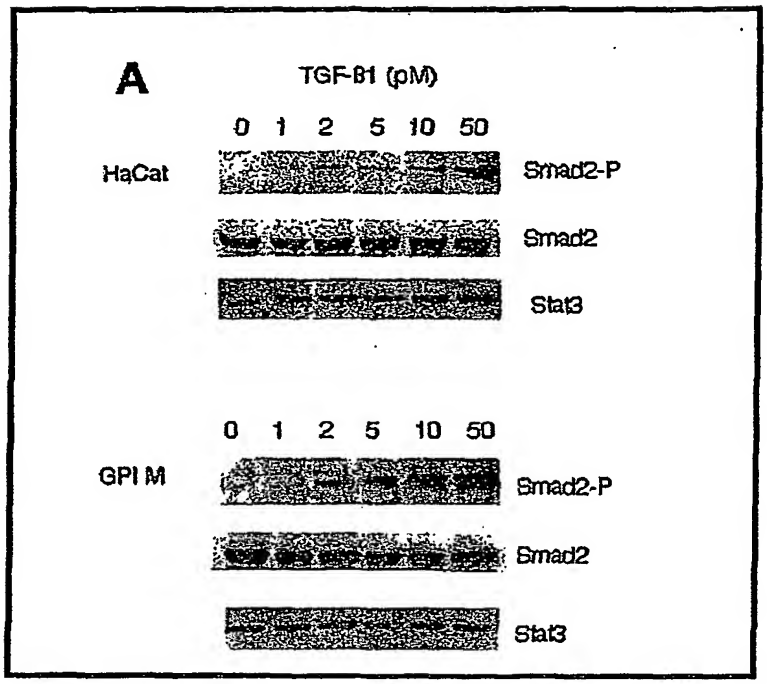


FIGURE 9 A

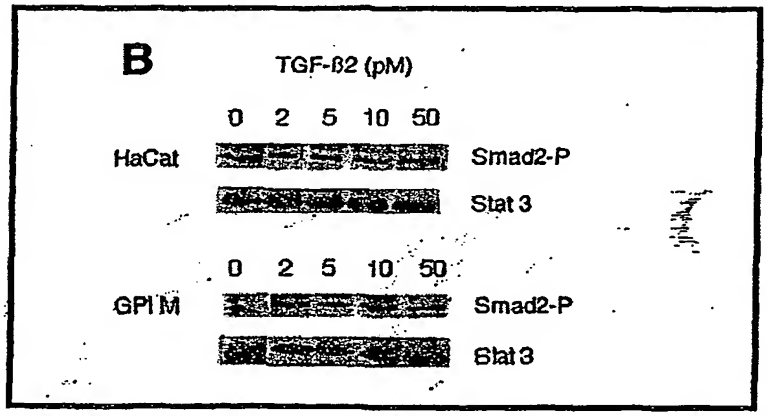


FIGURE 9 B

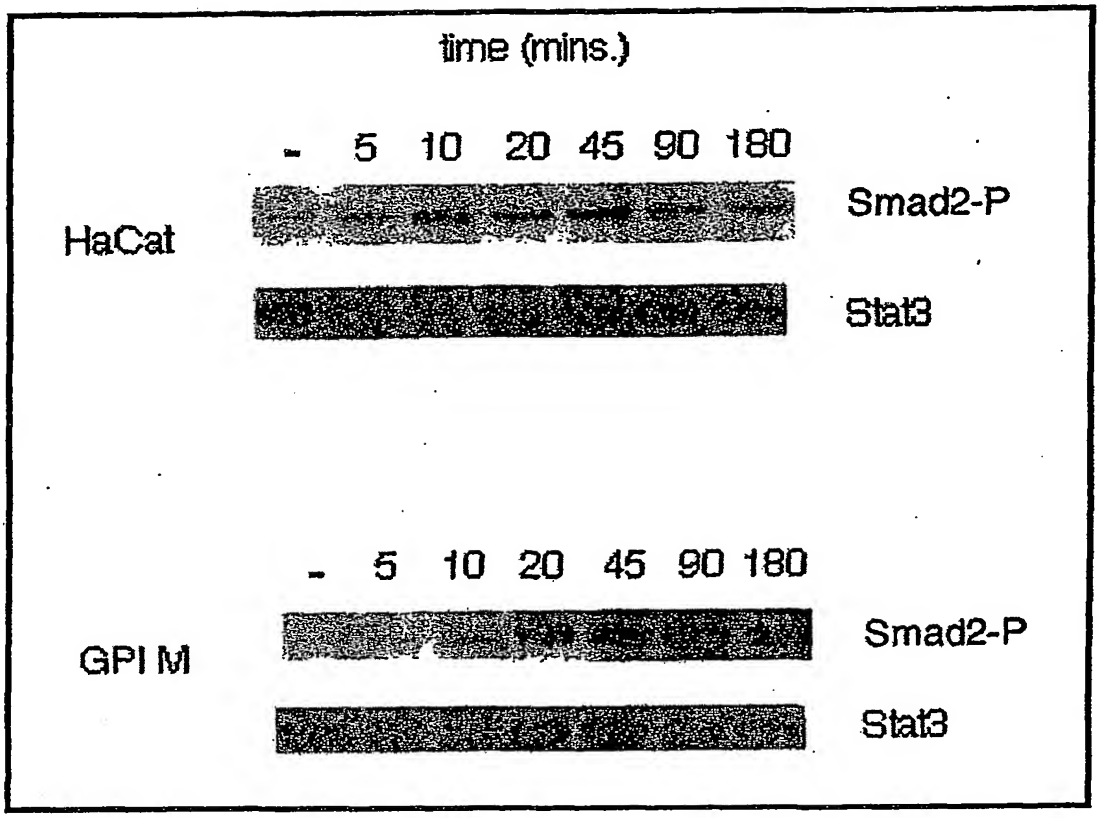


FIGURE 10 A

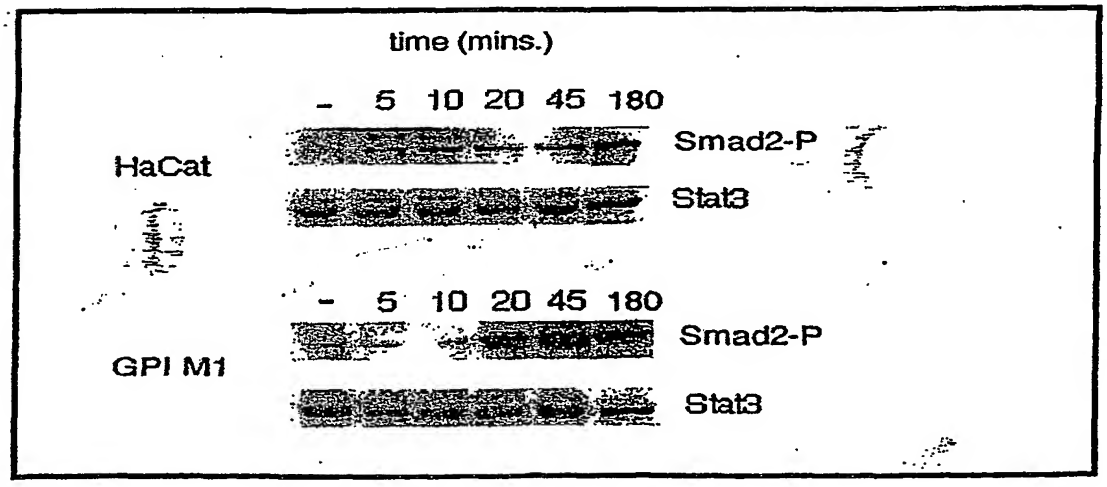


FIGURE 10 B

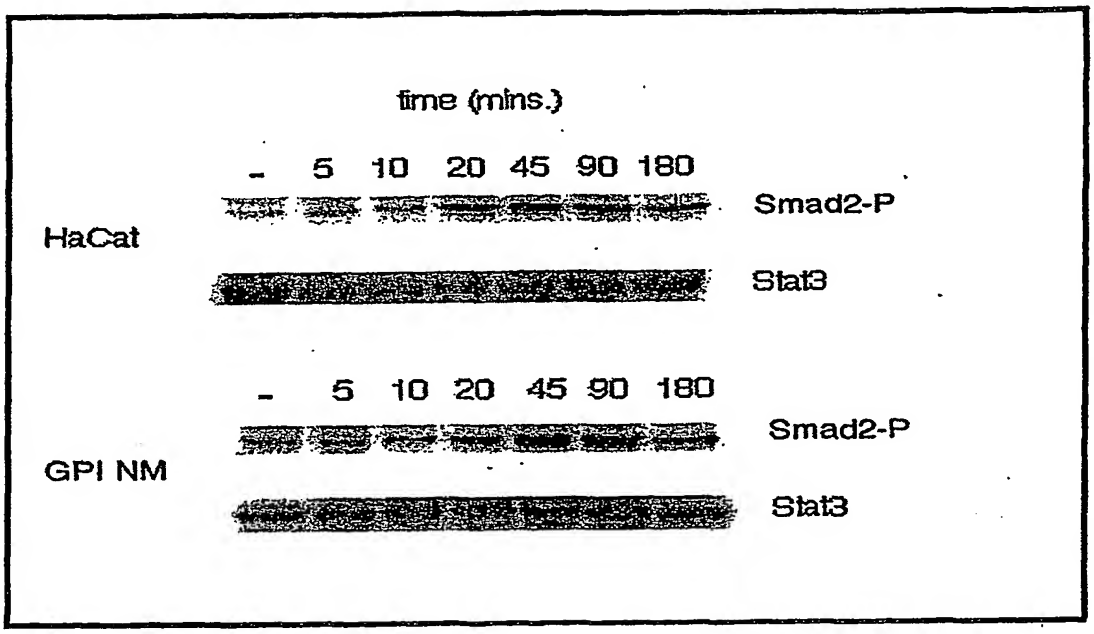


FIGURE 10 C

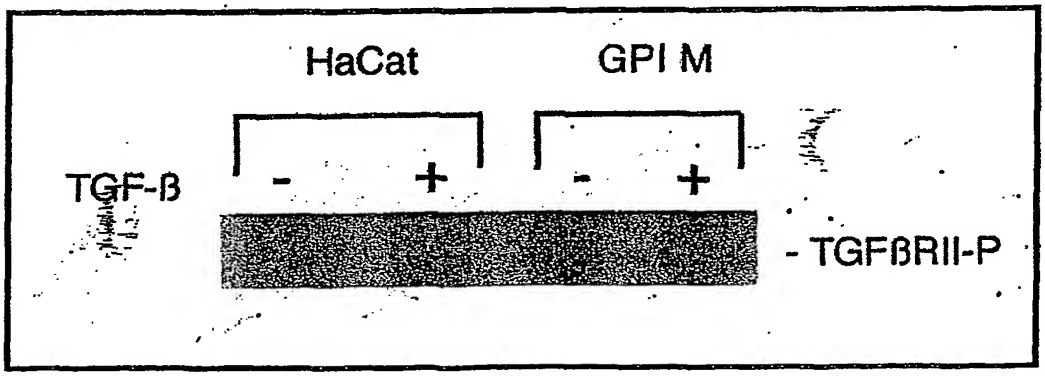
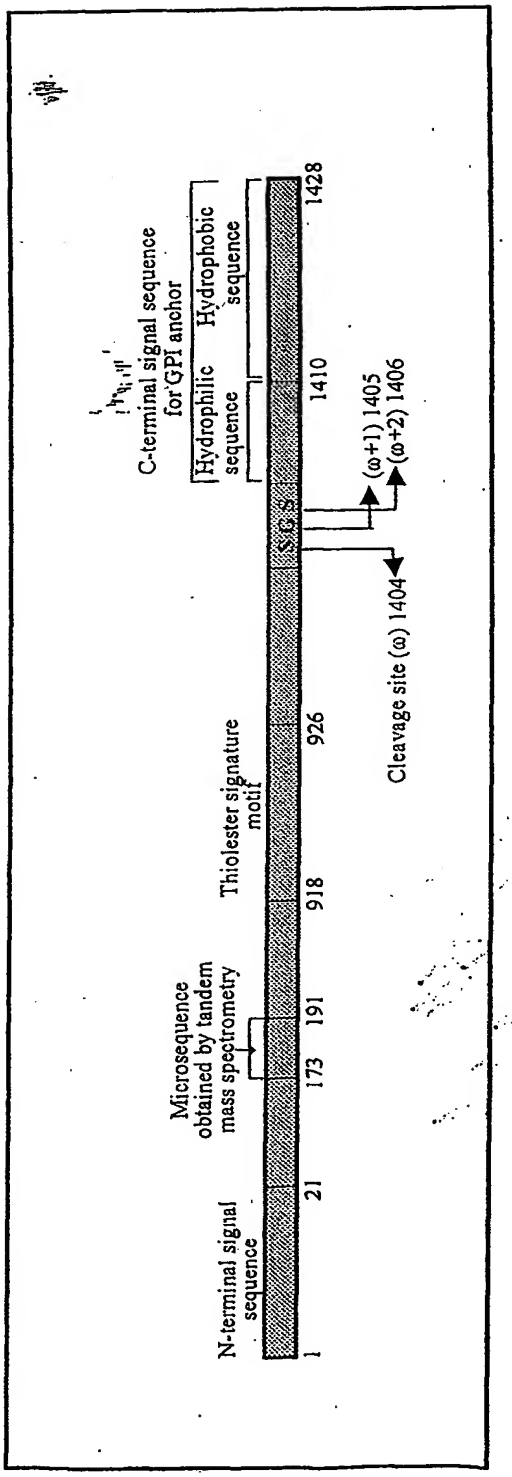
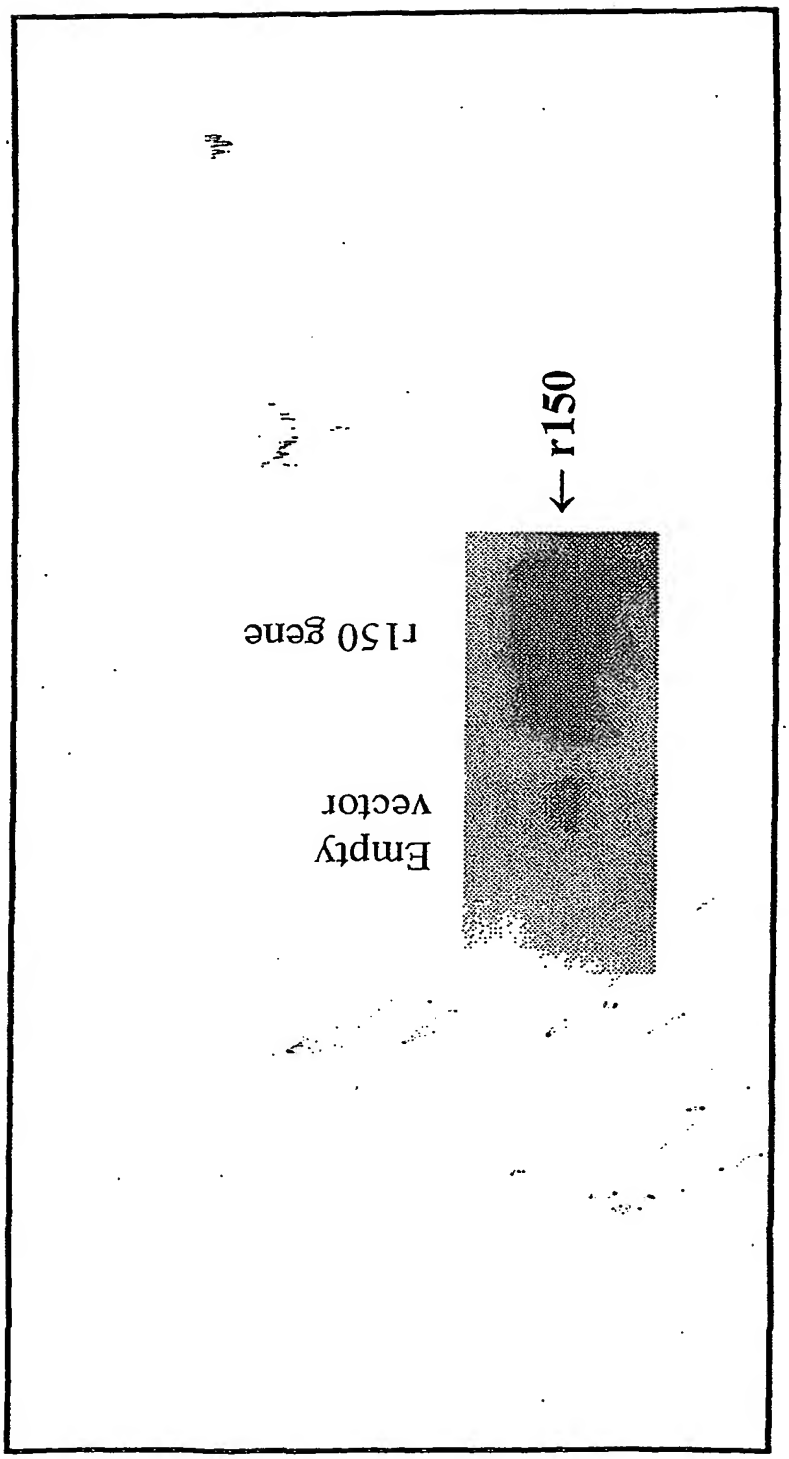


FIGURE 11



Schematic diagram representing the cloned sequence of the r150 protein.

FIGURE 12



Expression of the cloned gene in HaCaT cells demonstrates that it represents r150. The expressed protein migrates at 150 kDa on SDS-PAGE and is detectable by an antibody (anti-CRD antibody) which detects GPI-anchor.

FIGURE 13

Inhibition of TGFβ signaling by r150 : Expression of r150 markedly decreases TGFβ-induced Smad2 phosphorylation.

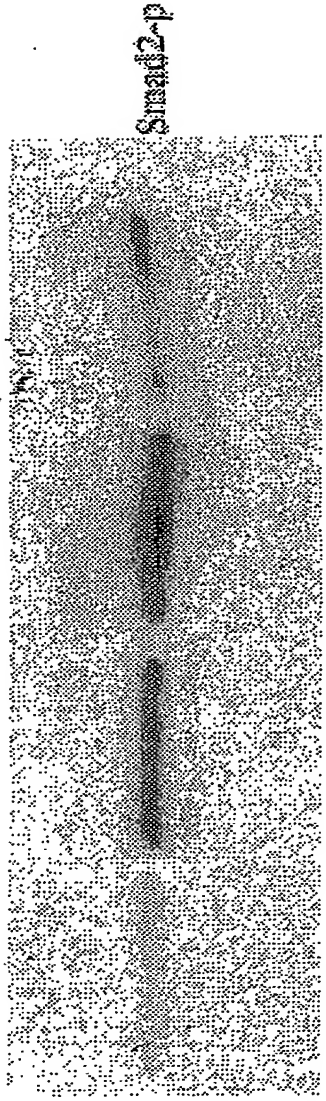


FIGURE 14

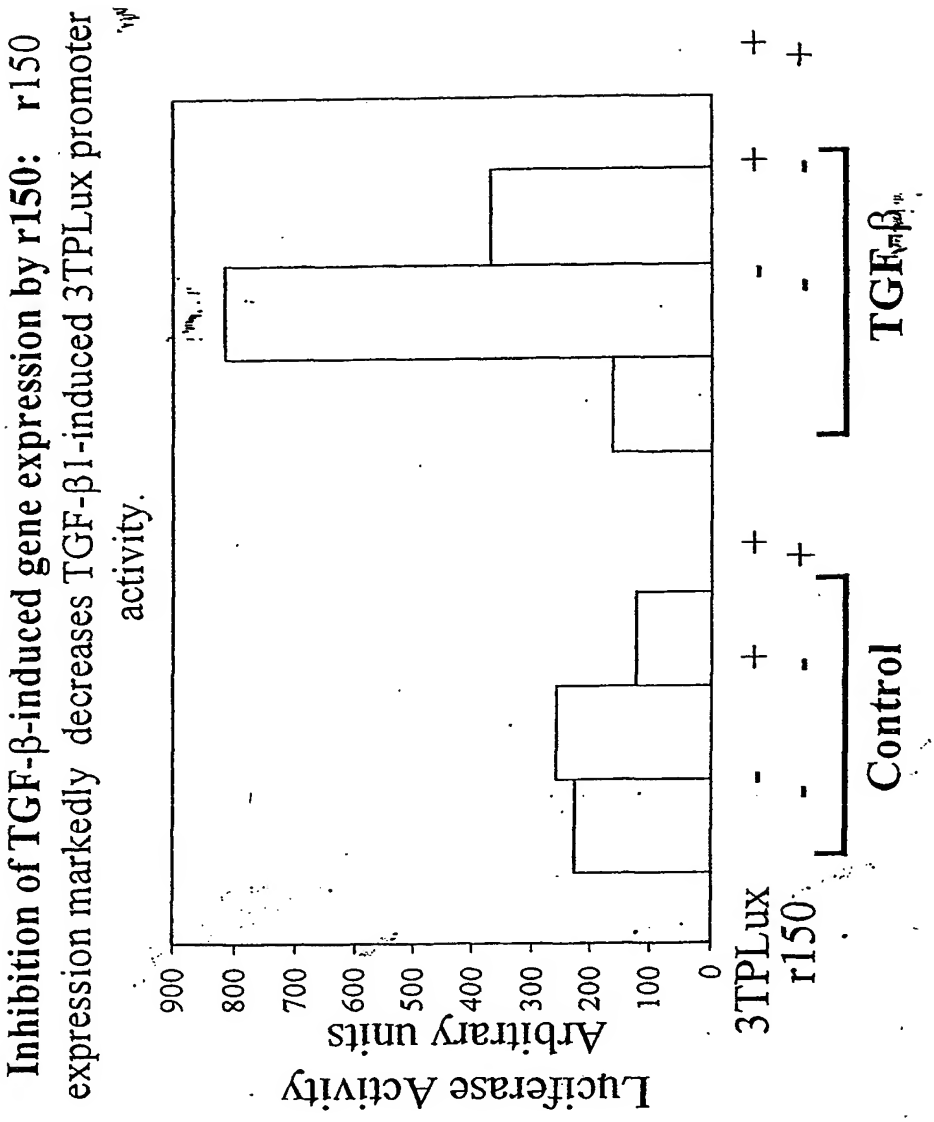

Inhibition of TGF-β-induced gene expression by r150: r150 expression markedly decreases TGF-β1-induced 3TPLux promoter activity.
Luciferase Activity
Arbitrary units
900
800
700
600
500
400
300
200
100
0
3TPLux
r150
Control
TGFβ

FIGURE 15

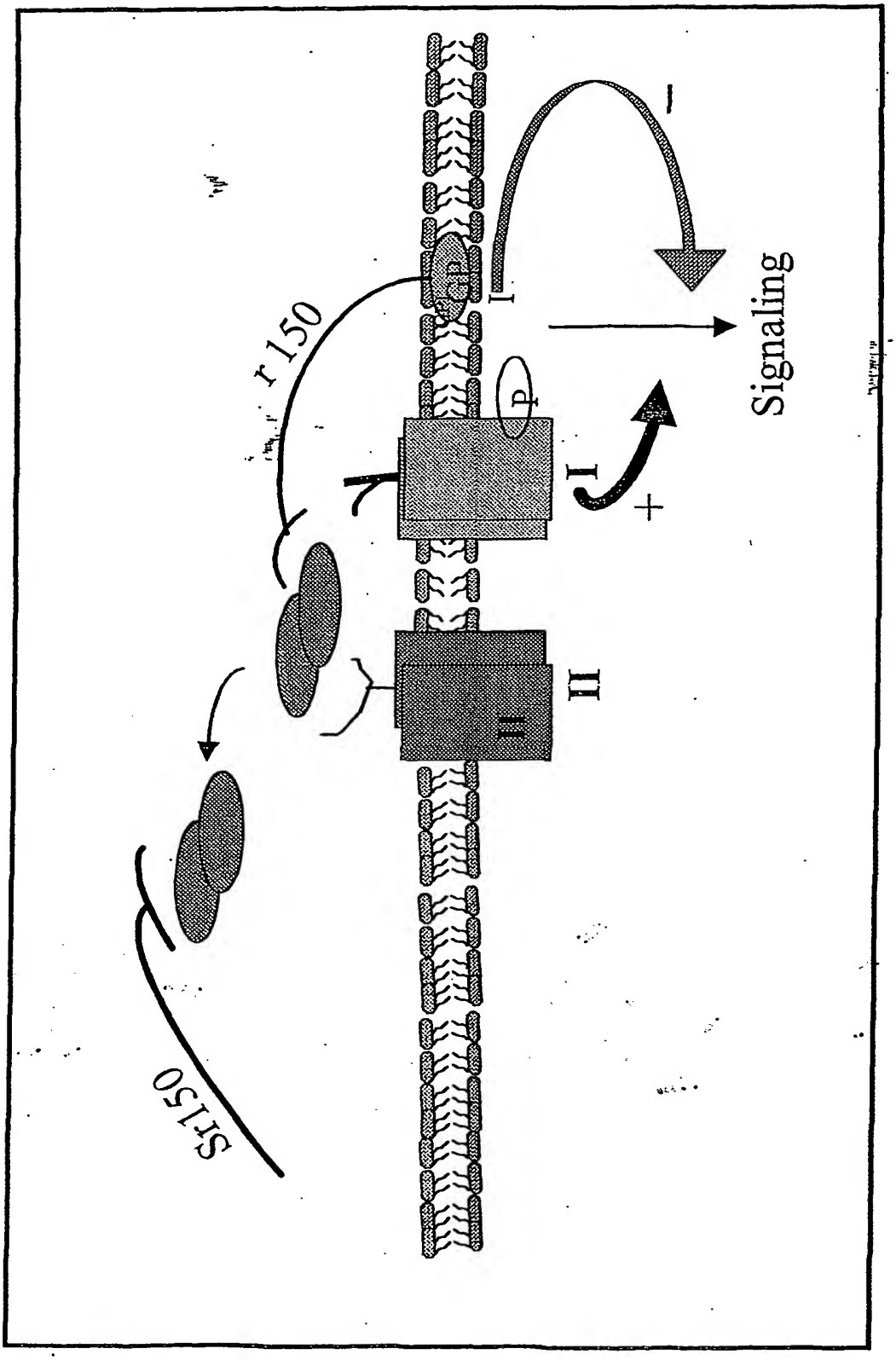

r150
sR150
I
II
P
P
I
II
+
−
Signaling
Both membrane anchored and soluble r150 regulate TGF-β response

FIGURE 16

```
-112                                                                ctaaact  -106
-105  cgaattaagagggaaaaaaaatcagggagtggcaagccacacccacggtgcccgcgaacttccccggcagcggactgtagcccaggcagacgccgtcgag    -1
   1  atgcagggcccaccgctcctgacccgccgcccacctcctctgcgtgtgcaccgccgctggccgtggctcccgggcctcggttctggtgacagcccccagggatc   105
 (2)    M  G  G  P  P  L  L  T  A  A  H  L  L  C  V  C  T  A  A  L  A  V  A  P  G  P  A  P  L  V  T  A  P  G  I   (35)
106  atcagccccggaggaaatgtgactattgggtggagcttctggaacactgccttcacagtgactgtgaaggcggagctgctcaagacagcatcaaacctcact   210
(36)    I  A  P  G  G  G  A  V  T  I  G  V  G  L  L  G  H  C  P  S  G  V  T  V  L  A  G  L  L  T  A  S  A  L  T   (70)
211  gtctctgtcctggaagcagaggagtctttgaaaaggctctttaagacacttactctccatcactacctctgaacagtgcagatgagatttatgagctacgt   315
(71)    V  S  V  L  G  A  G  G  V  P  G  T  G  S  P  L  T  L  T  L  P  S  L  P  L  A  S  A  A  G  I  T  G  L  A   (105)
316  gtaaccggacgtacccaggatgagatttatttctctaatagtaccgcttatcatttgagaccaagagaatatctgtcttcattcaaacagacaaggccttatac   420
(106)   V  T  G  A  T  G  A  G  I  L  P  S  A  S  T  A  L  S  P  G  T  L  A  I  S  V  P  I  G  T  A  L  T  T   (140)
421  aagccaaagcaagaagtgaagttcgcattgttacactcttctcagatttaagccttacaaaacctctttaaacattctcattaaggacccaaatcaaatttg   525
(141)   L  P  L  G  G  V  L  P  A  I  V  T  L  P  S  A  P  L  P  T  L  T  S  L  A  I  L  L  A  P  L  S  A  L   (175)
526  atccaacagtggttgtcacaacaaagtgatcttggagtcatttccaaaacttttcagctatcttcccatccaatacttggtgactggtctattcaagttcaagtg   630
(176)   I  G  G  T  L  S  G  G  S  A  L  G  V  I  S  L  T  P  G  L  S  S  H  P  I  L  G  A  T  S  I  G  V  G  V   (210)
631  aatgaccagacatattatcaatcatttcagtttcaggaatatgtattaccaaaatttgaagtgacttttgcagacaccattatattgttctatgaattctaagcat   735
(211)   A  A  G  T  T  T  G  S  P  G  V  S  G  T  V  L  P  L  P  G  V  T  L  G  T  P  L  T  C  S  M  A  S  L  H   (245)
736  ttaaatggtaccatcacggcaaagtatacatatgggaagccagtgaaaggagacgtaacgcttacatttttacctttatcctttgggaaagaagaaaaatatt   840
(246)   L  A  G  T  I  T  A  L  T  T  G  L  P  V  L  G  A  V  T  L  T  P  L  P  L  S  P  T  G  L  L  L  A  I   (280)
841  acaaaaacatttaagataaatggatctgcaaacttcctttaatgatgaagagatgaaaaatgtaatggattcttcaaatggacttttctgaatacctggatcta   945
(281)   T  L  T  P  L  I  A  G  S  A  A  P  S  P  A  A  G  G  M  L  A  V  M  A  S  S  A  G  L  S  G  T  L  A  L   (315)
946  tcttccctggaccagtagaaatttaaccacagtgacagaatcagttacaggtatttcaagaaatgtaagcactaatgtgttcttcaagcaacatgattacatc  1050
(316)   S  S  P  G  P  V  G  I  L  T  T  V  T  G  S  V  T  G  I  S  A  A  V  S  T  A  V  P  P  L  G  H  A  T  I   (350)
```

FIGURE 17

FIGURE 17 (CONT.)

FIGURE 17 (CONT.)

```
3361  atgcaattctgggtgtcatcagagtccaaacttctgactcctggcagccacgctccctggatattgaagttgcagcctatgcactgctctcacacttcttacaa  3465
(1121)  M G P T V S S G S L L S A S T G P A S L A I G V A A T A L L S H P L G                                    (1155)
3466  ttcagacttctgagggaatcccaattatgagtgctggctaagcagccaaagaaatagcttgggtgcttttgcatctactcaggataccactgtggctttaaaggct  3570
(1156)  P G T S G G G I P I M A T L S A G A A S L G G P A S T G A T T V A L L A                                    (1190)
3571  ctgtctgaatttgcagccctaatgaatacagaaatgacaaatatccaagtgaccgtgacgggcctagctcaccaagtcctgtaaagtttctgattgacacacac  3675
(1191)  L S G P A A L M A T G A T A I G V T V T G P S S P S P V L P L I A T H                                    (1225)
3676  aaccgcttactccttcagacagcagagcttgctgtgtacagccaatggcagttaatatttccgcaaatggtttggatttgctatttgtcagctcaatgttgtta  3780
(1226)  A A L L L G T A G L A V V G P M A V A I S A A G P G P A I C G L A V V                                    (1260)
3781  tataatgtgaaggcttctgggtcttctagaagacgaagatctatccaaaatcaagaagccttgattagatgttgctgtaaaagaaatataaagatgatctccaat  3885
(1261)  T A V L A S G S S A A A A S I G A G G A P A L A V A V L G A L A A L A                                    (1295)
3886  catgtggatttgaatgtgtgtacaagctttcgggcccgggtaggagtggcatggcttatggtggctaacctattaagtggctttatggtgccttcagaagca  3990
(1296)  H V A L A V C T S P S G P G A S G M A L M G V A L L S G P M V P S G A                                    (1330)
3991  atttctctgagcgagacagtgaagaaagtggaatatgatcatgtgaaaactcaacctatttagattctgtaaatgaaacccagtttgtgttaatattcctgct  4095
(1331)  I S L S G T V L L V G T A H G L L A L T L A S V A G T G P C V A I P A                                    (1365)
4096  gtgagaaactttaaagtttcaaatacccaagatgcttcagtgtccatagtggattactatgagccaagggagacagcggtgagaagttacaactctgaagtgaag  4200
(1366)  V A A P L V S A T G A A S V S I V A T T G P A A G A V A S T A S G V L                                    (1400)
4201  ctgtcctcctgtgacctttgcagtgatgtccagggctgccgtccttgtgaggatggagcttcaggctccatcactcttcagtcatttttatttctgttttc  4305
(1401)  L S S C A L C S A V G G C A P C G A G A S G S H H H S S V I P I P C P                                    (1435)
4306  aagcttctgtactttatggaacttggctgtga  4338
(1436)  L L L T P M G L T *
```

△

FIGURE 17 (CONT.)

```
4339 tttatttttaaaggactctgtgtaacactaacatttccagtagtcacatgtgattgttttgttttcgtagaa 4410
4411 gaatactgcttctatttttgaaaaaagagttttttttctttctatgggttgcagggatggtgtacaacaggtcctagcatgtatagctgcatagatttcttcacc 4515
4516 tgatctttgtgtggaagatcagaatgcagttgtgtgtctatatttccctcacaaaatctttagaatttttttggaggtgtttgttttctccagaataa 4620
4521 aggtattacttttagaa¦-----¦taggtattcctcctcatttttgtgaaagaaatgaacctagattcttaagcattattacacatccatgtttgcttaaagatggatttccct 4725
4637 gggaatgggagaaaacagccagcagaggagcttcatctgttcccttcccacctccaacctagcctactgcccacccacccccaacccacccatgcccagtgg 4830
4726 tctcagtagatacttcttaactggaaattcttcttttcagaatctaggtggtgaatttttttttaagtggcacggtctttttctgcttgaaatctgatcacaccc 4935
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5566 agggaaaaagtaattgccatgggctccaaagaatttgctttatgtttttagctatttaaaataaatccatcaaaaataaagtatgcaaatgtatctttt 5771
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FIGURE 17 (CONT.)

Comparison between our sequence and alpha 2 Macroglobulin (GI: 224053)

```
Identities = 63/197 (32%), Positives = 90/197 (46%), Gaps = 50/197 (25%)

Query: 621 YYLG---------------MFMNS--------------FAVFQECGLWVLTDANLTKDYI 651
           + G               +++N               ++  ++ GL   T++ + K  +
Sbjct: 605 GFPGPLNDQDDEDCINRHNVYINGITYTPVSSTNEKDMYSFLEDMGLKAFTNSKIRKPKM 664

Query: 652 DGVYDNAE-----------YAERFMEENEGHIVDIHDFSLGSSPH---VRKHFPETWIWL 697
               E           Y    M      +V + +       PH    VRK+FPETWIW
Sbjct: 665 CPQLQQYEMHGPEGLRVGFYESDVMGRGHARLVHVEE------PHTETVRKYFPETWIWD 718

Query: 698 DTNMGSRIYQEFEVTVPDSITSWVATGFVISEDLGLGLTTTPVELQAFQPFFIFLNLPYS 757
             + S    E  VTVPD+IT W A  F +SED GLG+++T   L+AFQPFF+ L +PYS
Sbjct: 719 LVVVNSAGVAEVGVTVPDTITEWKAGAFCLSEDAGLGISST-ASLRAFQPFFVELTMPYS 777

Query: 758 VIRGEEFALEITIFNYL 775
           VIRGE F L+ T+ NYL
Sbjct: 778 VIRGEAFTLKATVLNYL 795
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FIGURE 18

SEQUENCE LISTING

<110> McGill University, through its Office of Technology Transfer
PHILIP, Anie
TAM, Betty

<120> A 150 kDa accessory receptor for TGF-beta 1

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<140> PCT/CA02/00560
<141> 2002-04-24

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<151> 2001-04-24

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cccttcacag gtgactgtga aggcggagct gctcaagaca gcatcaaacc tcactgtctc 240

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actacctctg aacagtgcag atgagattta tgagctacgt gtaaccggac gtacccagga 360

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cattcaaaca gacaaggcct tatacaagcc aaagcaagaa gtgaagtttc gcattgttac 480

actcttctca gattttaagc cttacaaaac ctctttaaac attctcatta aggaccccaa 540

atcaaatttg atccaacagt ggttgtcaca acaaagtgat cttggagtca tttccaaaac 600

ttttcagcta tcttcccatc caatacttgg tgactggtct attcaagttc aagtgaatga 660

ccagacatac tatcaatcat ttcaggtttc agaatatgta ttaccaaaat ttgaagtgac 720

tttgcagaca ccattatatt gttctatgaa ttctaagcat ttaaatggta ccatcacggc 780

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tggtgtttat gacaatgcag aatatgctga gaggtttatg gaggaaaatg aaggacatat 2040

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Leu Glu His Cys Pro Ser Gln Val Thr Val Lys Ala Glu Leu Leu Lys
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Thr Ala Ser Asn Leu Thr Val Ser Val Leu Glu Ala Glu Gly Val Phe
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Glu Lys Gly Ser Phe Lys Thr Leu Thr Leu Pro Ser Leu Pro Leu Asn
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Ser Ala Asp Glu Ile Tyr Glu Leu Arg Val Thr Gly Arg Thr Gln Asp
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Glu Ile Leu Phe Ser Asn Ser Thr Arg Leu Ser Phe Glu Thr Lys Arg
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Ile Ser Val Phe Ile Gln Thr Asp Lys Ala Leu Tyr Lys Pro Lys Gln
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Glu Val Lys Phe Arg Ile Val Thr Leu Phe Ser Asp Phe Lys Pro Tyr
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Lys Thr Ser Leu Asn Ile Leu Ile Lys Asp Pro Lys Ser Asn Leu Ile
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Phe Gln Leu Ser Ser His Pro Ile Leu Gly Asp Trp Ser Ile Gln Val
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690 695 700

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740 745 750

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Arg Ala  Glu Gln Glu Gly Gly  Met Gln Phe Trp Val  Ser Ser Glu
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Ser Lys  Leu Ser Asp Ser Trp  Gln Pro Arg Ser Leu  Asp Ile Glu
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Val Ala  Ala Tyr Ala Leu Leu  Ser His Phe Leu Gln  Phe Gln Thr
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Ser Glu  Gly Ile Pro Ile Met  Arg Trp Leu Ser Arg  Gln Arg Asn
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His Val Asp Leu Asn Val Cys Thr Ser Phe Ser Gly Pro Gly Arg
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Ser Gly Met Ala Leu Met Glu Val Asn Leu Leu Ser Gly Phe Met
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Glu Thr Gln Phe Cys Val Asn Ile Pro Ala Val Arg Asn Phe Lys
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Glu Pro Arg Arg Gln Ala Val Arg Ser Tyr Asn Ser Glu Val Lys
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Ser Gln Val Thr Val Lys Ala Glu Leu Leu Lys Thr Ala Ser Asn Leu
35 40 45

Thr Val Ser Val Leu Glu Ala Glu Gly Val Phe Glu Lys Gly Ser Phe
50 55 60

Lys Thr Leu Thr Leu Pro Ser Leu Pro Leu Asn Ser Ala Asp Glu Ile
65 70 75 80

Tyr Glu Leu Arg Val Thr Gly Arg Thr Gln Asp Glu Ile Leu Phe Ser
85 90 95

Asn Ser Thr Arg Leu Ser Phe Glu Thr Lys Arg Ile Ser Val Phe Ile
100 105 110

Gln Thr Asp Lys Ala Leu Tyr Lys Pro Lys Gln Glu Val Lys Phe Arg
115 120 125

Ile Val Thr Leu Phe Ser Asp Phe Lys Pro Tyr Lys Thr Ser Leu Asn
130 135 140

Ile Leu Ile Lys Asp Pro Lys Ser Asn Leu Ile Gln Gln Trp Leu Ser
145 150 155 160

Gln Gln Ser Asp Leu Gly Val Ile Ser Lys Thr Phe Gln Leu Ser Ser
165 170 175

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His Pro Ile Leu Gly Asp Trp Ser Ile Gln Val Gln Val Asn Asp Gln
            180                 185                 190

Thr Tyr Tyr Gln Ser Phe Gln Val Ser Glu Tyr Val Leu Pro Lys Phe
        195                 200                 205

Glu Val Thr Leu Gln Thr Pro Leu Tyr Cys Ser Met Asn Ser Lys His
    210                 215                 220

Leu Asn Gly Thr Ile Thr Ala Lys Tyr Thr Tyr Gly Lys Pro Val Lys
225                 230                 235                 240

Gly Asp Val Thr Leu Thr Phe Leu Pro Leu Ser Phe Trp Gly Lys Lys
                245                 250                 255

Lys Asn Ile Thr Lys Thr Phe Lys Ile Asn Gly Ser Ala Asn Phe Ser
            260                 265                 270

Phe Asn Asp Glu Glu Met Lys Asn Val Met Asp Ser Ser Asn Gly Leu
        275                 280                 285

Ser Glu Tyr Leu Asp Leu Ser Ser Pro Gly Pro Val Glu Ile Leu Thr
    290                 295                 300

Thr Val Thr Glu Ser Val Thr Gly Ile Ser Arg Asn Val Ser Thr Asn
305                 310                 315                 320

Val Phe Phe Lys Gln His Asp Tyr Ile Ile Glu Phe Phe Asp Tyr Thr
                325                 330                 335

Thr Val Leu Lys Pro Ser Leu Asn Phe Thr Ala Thr Val Lys Val Thr
            340                 345                 350

Arg Ala Asp Gly Asn Gln Leu Thr Leu Glu Glu Arg Arg Asn Asn Val
        355                 360                 365

Val Ile Thr Val Thr Gln Arg Asn Tyr Thr Glu Tyr Trp Ser Gly Ser
    370                 375                 380

Asn Ser Gly Asn Gln Lys Met Glu Ala Val Gln Lys Ile Asn Tyr Thr
385                 390                 395                 400

Val Pro Gln Ser Gly Thr Phe Lys Ile Glu Phe Pro Ile Leu Glu Asp
                405                 410                 415

Ser Ser Glu Leu Gln Leu Lys Ala Tyr Phe Leu Gly Ser Lys Ser Ser
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420 425 430

Met Ala Val His Ser Leu Phe Lys Ser Pro Ser Lys Thr Tyr Ile Gln
435 440 445

Leu Lys Thr Arg Asp Glu Asn Ile Lys Val Gly Ser Pro Phe Glu Leu
450 455 460

Val Val Ser Gly Asn Lys Arg Leu Lys Glu Leu Ser Tyr Met Val Val
465 470 475 480

Ser Arg Gly Gln Leu Val Ala Val Gly Lys Gln Asn Ser Thr Met Phe
485 490 495

Ser Leu Thr Pro Glu Asn Ser Trp Thr Pro Lys Ala Cys Val Ile Val
500 505 510

Tyr Tyr Ile Glu Asp Asp Gly Glu Ile Ile Ser Asp Val Leu Lys Ile
515 520 525

Pro Val Gln Leu Val Phe Lys Asn Lys Ile Lys Leu Tyr Trp Ser Lys
530 535 540

Val Lys Ala Glu Pro Ser Glu Lys Val Ser Leu Arg Ile Ser Val Thr
545 550 555 560

Gln Pro Asp Ser Ile Val Gly Ile Val Ala Val Asp Lys Ser Val Asn
565 570 575

Leu Met Asn Ala Ser Asn Asp Ile Thr Met Glu Asn Val Val His Glu
580 585 590

Leu Glu Leu Tyr Asn Thr Gly Tyr Tyr Leu Gly Met Phe Met Asn Ser
595 600 605

Phe Ala Val Phe Gln Glu Cys Gly Leu Trp Val Leu Thr Asp Ala Asn
610 615 620

Leu Thr Lys Asp Tyr Ile Asp Gly Val Tyr Asp Asn Ala Glu Tyr Ala
625 630 635 640

Glu Arg Phe Met Glu Glu Asn Glu Gly His Ile Val Asp Ile His Asp
645 650 655

Phe Ser Leu Gly Ser Ser Pro His Val Arg Lys His Phe Pro Glu Thr
660 665 670

Trp Ile Trp Leu Asp Thr Asn Met Gly Xaa Arg Ile Tyr Gln Glu Phe
675 680 685

Glu Val Thr Val Pro Asp Ser Ile Thr Ser Trp Val Ala Thr Gly Phe
690 695 700

Val Ile Ser Glu Asp Leu Gly Leu Gly Leu Thr Thr Thr Pro Val Glu
705 710 715 720

Leu Gln Ala Phe Gln Pro Phe Phe Ile Phe Leu Asn Leu Pro Tyr Ser
725 730 735

Val Ile Arg Gly Glu Glu Phe Ala Leu Glu Ile Thr Ile Phe Asn Tyr
740 745 750

Leu Lys Asp Ala Thr Glu Val Lys Val Ile Ile Glu Lys Ser Asp Lys
755 760 765

Phe Asp Ile Leu Met Thr Ser Asn Glu Ile Asn Ala Thr Gly His Gln
770 775 780

Gln Thr Leu Leu Val Pro Ser Glu Asp Gly Ala Thr Val Leu Phe Pro
785 790 795 800

Ile Arg Pro Thr His Leu Gly Glu Ile Pro Ile Thr Val Thr Ala Leu
805 810 815

Ser Pro Thr Ala Ser Asp Ala Val Thr Gln Met Ile Leu Val Lys Ala
820 825 830

Glu Gly Ile Glu Lys Ser Tyr Ser Gln Ser Ile Leu Leu Asp Leu Thr
835 840 845

Asp Asn Arg Leu Gln Ser Thr Leu Lys Thr Leu Ser Phe Ser Phe Pro
850 855 860

Pro Asn Thr Val Thr Gly Ser Glu Arg Val Gln Ile Thr Ala Ile Gly
865 870 875 880

Asp Val Leu Gly Pro Ser Ile Asn Gly Leu Ala Ser Leu Ile Arg Met
885 890 895

Pro Tyr Gly Cys Gly Glu Gln Asn Met Ile Asn Phe Ala Pro Asn Ile
900 905 910

Tyr Ile Leu Asp Tyr Leu Thr Lys Lys Lys Gln Leu Thr Asp Asn Leu

915 920 925

Lys Glu Lys Ala Leu Ser Phe Met Arg Gln Gly Tyr Gln Arg Glu Leu
930 935 940

Leu Tyr Gln Arg Glu Asp Gly Ser Phe Ser Ala Phe Gly Asn Tyr Asp
945 950 955 960

Pro Ser Gly Ser Thr Trp Leu Ser Ala Phe Val Leu Arg Cys Phe Leu
965 970 975

Glu Ala Asp Pro Tyr Ile Asp Ile Asp Gln Asn Val Leu His Arg Thr
980 985 990

Tyr Thr Trp Leu Lys Gly His Gln Lys Ser Asn Gly Glu Phe Trp Asp
995 1000 1005

Pro Gly Arg Val Ile His Ser Glu Leu Gln Gly Gly Asn Lys Ser
1010 1015 1020

Pro Val Thr Leu Thr Ala Tyr Ile Val Thr Ser Leu Leu Gly Tyr
1025 1030 1035

Arg Lys Tyr Gln Pro Asn Ile Asp Val Gln Glu Ser Ile His Phe
1040 1045 1050

Leu Glu Ser Glu Phe Ser Arg Gly Ile Ser Asp Asn Tyr Thr Leu
1055 1060 1065

Ala Leu Ile Thr Tyr Ala Leu Ser Ser Val Gly Ser Pro Lys Ala
1070 1075 1080

Lys Glu Ala Leu Asn Met Leu Thr Trp Arg Ala Glu Gln Glu Gly
1085 1090 1095

Gly Met Gln Phe Trp Val Ser Ser Glu Ser Lys Leu Ser Asp Ser
1100 1105 1110

Trp Gln Pro Arg Ser Leu Asp Ile Glu Val Ala Ala Tyr Ala Leu
1115 1120 1125

Leu Ser His Phe Leu Gln Phe Gln Thr Ser Glu Gly Ile Pro Ile
1130 1135 1140

Met Arg Trp Leu Ser Arg Gln Arg Asn Ser Leu Gly Gly Phe Ala
1145 1150 1155

Ser Thr Gln Asp Thr Thr Val Ala Leu Lys Ala Leu Ser Glu Phe
1160 1165 1170

Ala Ala Leu Met Asn Thr Glu Arg Thr Asn Ile Gln Val Thr Val
1175 1180 1185

Thr Gly Pro Ser Ser Pro Ser Pro Leu Ala Val Val Gln Pro Thr
1190 1195 1200

Ala Val Asn Ile Ser Ala Asn Gly Phe Gly Phe Ala Ile Cys Gln
1205 1210 1215

Leu Asn Val Val Tyr Asn Val Lys Ala Ser Gly Ser Ser Arg Arg
1220 1225 1230

Arg Arg Ser Ile Gln Asn Gln Glu Ala Phe Asp Leu Asp Val Ala
1235 1240 1245

Val Lys Glu Asn Lys Asp Asp Leu Asn His Val Asp Leu Asn Val
1250 1255 1260

Cys Thr Ser Phe Ser Gly Pro Gly Arg Ser Gly Met Ala Leu Met
1265 1270 1275

Glu Val Asn Leu Leu Ser Gly Phe Met Val Pro Ser Glu Ala Ile
1280 1285 1290

Ser Leu Ser Glu Thr Val Lys Lys Val Glu Tyr Asp His Gly Lys
1295 1300 1305

Leu Asn Leu Tyr Leu Asp Ser Val Asn Glu Thr Gln Phe Cys Val
1310 1315 1320

Asn Ile Pro Ala Val Arg Asn Phe Lys Val Ser Asn Thr Gln Asp
1325 1330 1335

Ala Ser Val Ser Ile Val Asp Tyr Tyr Glu Pro Arg Arg Gln Ala
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Val Arg Ser Tyr Asn Ser Glu Val Lys Leu Ser Ser Cys Asp Leu
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Cys Ser Asp Val Gln Gly Cys Arg Pro Cys Glu Asp Gly Ala
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<211> 4197
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gtggattact atgagccaag gagacaggcg gtgagaagtt acaactctga agtgaagctg 4140

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Ser Gln Val Thr Val Lys Ala Glu Leu Leu Lys Thr Ala Ser Asn Leu
35 40 45

Thr Val Ser Val Leu Glu Ala Glu Gly Val Phe Glu Lys Gly Ser Phe
50 55 60

Lys Thr Leu Thr Leu Pro Ser Leu Pro Leu Asn Ser Ala Asp Glu Ile
65 70 75 80

Tyr Glu Leu Arg Val Thr Gly Arg Thr Gln Asp Glu Ile Leu Phe Ser

85 90 95

Asn Ser Thr Arg Leu Ser Phe Glu Thr Lys Arg Ile Ser Val Phe Ile
100 105 110

Gln Thr Asp Lys Ala Leu Tyr Lys Pro Lys Gln Glu Val Lys Phe Arg
115 120 125

Ile Val Thr Leu Phe Ser Asp Phe Lys Pro Tyr Lys Thr Ser Leu Asn
130 135 140

Ile Leu Ile Lys Asp Pro Lys Ser Asn Leu Ile Gln Gln Trp Leu Ser
145 150 155 160

Gln Gln Ser Asp Leu Gly Val Ile Ser Lys Thr Phe Gln Leu Ser Ser
165 170 175

His Pro Ile Leu Gly Asp Trp Ser Ile Gln Val Gln Val Asn Asp Gln
180 185 190

Thr Tyr Tyr Gln Ser Phe Gln Val Ser Glu Tyr Val Leu Pro Lys Phe
195 200 205

Glu Val Thr Leu Gln Thr Pro Leu Tyr Cys Ser Met Asn Ser Lys His
210 215 220

Leu Asn Gly Thr Ile Thr Ala Lys Tyr Thr Tyr Gly Lys Pro Val Lys
225 230 235 240

Gly Asp Val Thr Leu Thr Phe Leu Pro Leu Ser Phe Trp Gly Lys Lys
245 250 255

Lys Asn Ile Thr Lys Thr Phe Lys Ile Asn Gly Ser Ala Asn Phe Ser
260 265 270

Phe Asn Asp Glu Glu Met Lys Asn Val Met Asp Ser Ser Asn Gly Leu
275 280 285

Ser Glu Tyr Leu Asp Leu Ser Ser Pro Gly Pro Val Glu Ile Leu Thr
290 295 300

Thr Val Thr Glu Ser Val Thr Gly Ile Ser Arg Asn Val Ser Thr Asn
305 310 315 320

Val Phe Phe Lys Gln His Asp Tyr Ile Ile Glu Phe Phe Asp Tyr Thr
325 330 335

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Thr Val Leu Lys Pro Ser Leu Asn Phe Thr Ala Thr Val Lys Val Thr
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Arg Ala Asp Gly Asn Gln Leu Thr Leu Glu Glu Arg Arg Asn Asn Val
        355                 360                 365

Val Ile Thr Val Thr Gln Arg Asn Tyr Thr Glu Tyr Trp Ser Gly Ser
    370                 375                 380

Asn Ser Gly Asn Gln Lys Met Glu Ala Val Gln Lys Ile Asn Tyr Thr
385                 390                 395                 400

Val Pro Gln Ser Gly Thr Phe Lys Ile Glu Phe Pro Ile Leu Glu Asp
                405                 410                 415

Ser Ser Glu Leu Gln Leu Lys Ala Tyr Phe Leu Gly Ser Lys Ser Ser
            420                 425                 430

Met Ala Val His Ser Leu Phe Lys Ser Pro Ser Lys Thr Tyr Ile Gln
        435                 440                 445

Leu Lys Thr Arg Asp Glu Asn Ile Lys Val Gly Ser Pro Phe Glu Leu
    450                 455                 460

Val Val Ser Gly Asn Lys Arg Leu Lys Glu Leu Ser Tyr Met Val Val
465                 470                 475                 480

Ser Arg Gly Gln Leu Val Ala Val Gly Lys Gln Asn Ser Thr Met Phe
                485                 490                 495

Ser Leu Thr Pro Glu Asn Ser Trp Thr Pro Lys Ala Cys Val Ile Val
            500                 505                 510

Tyr Tyr Ile Glu Asp Asp Gly Glu Ile Ile Ser Asp Val Leu Lys Ile
        515                 520                 525

Pro Val Gln Leu Val Phe Lys Asn Lys Ile Lys Leu Tyr Trp Ser Lys
    530                 535                 540

Val Lys Ala Glu Pro Ser Glu Lys Val Ser Leu Arg Ile Ser Val Thr
545                 550                 555                 560

Gln Pro Asp Ser Ile Val Gly Ile Val Ala Val Asp Lys Ser Val Asn
                565                 570                 575

Leu Met Asn Ala Ser Asn Asp Ile Thr Met Glu Asn Val Val His Glu
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580 585 590

Leu Glu Leu Tyr Asn Thr Gly Tyr Tyr Leu Gly Met Phe Met Asn Ser
595 600 605

Phe Ala Val Phe Gln Glu Cys Gly Leu Trp Val Leu Thr Asp Ala Asn
610 615 620

Leu Thr Lys Asp Tyr Ile Asp Gly Val Tyr Asp Asn Ala Glu Tyr Ala
625 630 635 640

Glu Arg Phe Met Glu Glu Asn Glu Gly His Ile Val Asp Ile His Asp
645 650 655

Phe Ser Leu Gly Ser Ser Pro His Val Arg Lys His Phe Pro Glu Thr
660 665 670

Trp Ile Trp Leu Asp Thr Asn Met Gly Xaa Arg Ile Tyr Gln Glu Phe
675 680 685

Glu Val Thr Val Pro Asp Ser Ile Thr Ser Trp Val Ala Thr Gly Phe
690 695 700

Val Ile Ser Glu Asp Leu Gly Leu Gly Leu Thr Thr Thr Pro Val Glu
705 710 715 720

Leu Gln Ala Phe Gln Pro Phe Phe Ile Phe Leu Asn Leu Pro Tyr Ser
725 730 735

Val Ile Arg Gly Glu Glu Phe Ala Leu Glu Ile Thr Ile Phe Asn Tyr
740 745 750

Leu Lys Asp Ala Thr Glu Val Lys Val Ile Ile Glu Lys Ser Asp Lys
755 760 765

Phe Asp Ile Leu Met Thr Ser Asn Glu Ile Asn Ala Thr Gly His Gln
770 775 780

Gln Thr Leu Leu Val Pro Ser Glu Asp Gly Ala Thr Val Leu Phe Pro
785 790 795 800

Ile Arg Pro Thr His Leu Gly Glu Ile Pro Ile Thr Val Thr Ala Leu
805 810 815

Ser Pro Thr Ala Ser Asp Ala Val Thr Gln Met Ile Leu Val Lys Ala
820 825 830

Glu Gly Ile Glu Lys Ser Tyr Ser Gln Ser Ile Leu Leu Asp Leu Thr
835 840 845

Asp Asn Arg Leu Gln Ser Thr Leu Lys Thr Leu Ser Phe Ser Phe Pro
850 855 860

Pro Asn Thr Val Thr Gly Ser Glu Arg Val Gln Ile Thr Ala Ile Gly
865 870 875 880

Asp Val Leu Gly Pro Ser Ile Asn Gly Leu Ala Ser Leu Ile Arg Met
885 890 895

Pro Tyr Gly Cys Gly Glu Gln Asn Met Ile Asn Phe Ala Pro Asn Ile
900 905 910

Tyr Ile Leu Asp Tyr Leu Thr Lys Lys Lys Gln Leu Thr Asp Asn Leu
915 920 925

Lys Glu Lys Ala Leu Ser Phe Met Arg Gln Gly Tyr Gln Arg Glu Leu
930 935 940

Leu Tyr Gln Arg Glu Asp Gly Ser Phe Ser Ala Phe Gly Asn Tyr Asp
945 950 955 960

Pro Ser Gly Ser Thr Trp Leu Ser Ala Phe Val Leu Arg Cys Phe Leu
965 970 975

Glu Ala Asp Pro Tyr Ile Asp Ile Asp Gln Asn Val Leu His Arg Thr
980 985 990

Tyr Thr Trp Leu Lys Gly His Gln Lys Ser Asn Gly Glu Phe Trp Asp
995 1000 1005

Pro Gly Arg Val Ile His Ser Glu Leu Gln Gly Gly Asn Lys Ser
1010 1015 1020

Pro Val Thr Leu Thr Ala Tyr Ile Val Thr Ser Leu Leu Gly Tyr
1025 1030 1035

Arg Lys Tyr Gln Pro Asn Ile Asp Val Gln Glu Ser Ile His Phe
1040 1045 1050

Leu Glu Ser Glu Phe Ser Arg Gly Ile Ser Asp Asn Tyr Thr Leu
1055 1060 1065

Ala Leu Ile Thr Tyr Ala Leu Ser Ser Val Gly Ser Pro Lys Ala

1070 1075 1080

Lys Glu Ala Leu Asn Met Leu Thr Trp Arg Ala Glu Gln Glu Gly
1085 1090 1095

Gly Met Gln Phe Trp Val Ser Ser Glu Ser Lys Leu Ser Asp Ser
1100 1105 1110

Trp Gln Pro Arg Ser Leu Asp Ile Glu Val Ala Ala Tyr Ala Leu
1115 1120 1125

Leu Ser His Phe Leu Gln Phe Gln Thr Ser Glu Gly Ile Pro Ile
1130 1135 1140

Met Arg Trp Leu Ser Arg Gln Arg Asn Ser Leu Gly Gly Phe Ala
1145 1150 1155

Ser Thr Gln Asp Thr Thr Val Ala Leu Lys Ala Leu Ser Glu Phe
1160 1165 1170

Ala Ala Leu Met Asn Thr Glu Arg Thr Asn Ile Gln Val Thr Val
1175 1180 1185

Thr Gly Pro Ser Ser Pro Ser Pro Val Lys Phe Leu Ile Asp Thr
1190 1195 1200

His Asn Arg Leu Leu Leu Gln Thr Ala Glu Leu Ala Val Val Gln
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Pro Met Ala Val Asn Ile Ser Ala Asn Gly Phe Gly Phe Ala Ile
1220 1225 1230

Cys Gln Leu Asn Val Val Tyr Asn Val Lys Ala Ser Gly Ser Ser
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Arg Arg Arg Arg Ser Ile Gln Asn Gln Glu Ala Phe Asp Leu Asp
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Val Ala Val Lys Glu Asn Lys Asp Asp Leu Asn His Val Asp Leu
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Asn Val Cys Thr Ser Phe Ser Gly Pro Gly Arg Ser Gly Met Ala
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Leu Met Glu Val Asn Leu Leu Ser Gly Phe Met Val Pro Ser Glu
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Ala Ile Ser Leu Ser Glu Thr Val Lys Lys Val Glu Tyr Asp His
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Gly Lys Leu Asn Leu Tyr Leu Asp Ser Val Asn Glu Thr Gln Phe
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Cys Val Asn Ile Pro Ala Val Arg Asn Phe Lys Val Ser Asn Thr
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Gln Asp Ala Ser Val Ser Ile Val Asp Tyr Tyr Glu Pro Arg Arg
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Gln Ala Val Arg Ser Tyr Asn Ser Glu Val Lys Leu Ser Ser Cys
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Ala

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Glu Val Thr